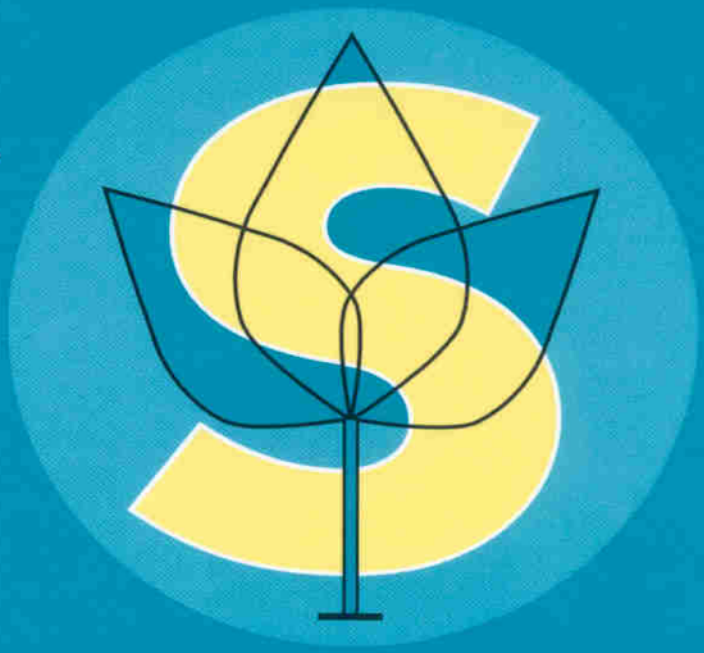
# Sulphur metabolism in higher plants

## molecular, ecophysiological and nutritional aspects




editors: W.J.Cram - L.J. De Kok - I.Stulen - C.Brunold - H.Rennenberg

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## MOLECULAR, ECOPHYSIOLOGICAL AND NUTRITIONAL ASPECTS

Edited by W.J. Cram, L.J. De Kok, I. Stulen, C. Brunold and H. Rennenberg

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**Contributed papers**

## PREFACE

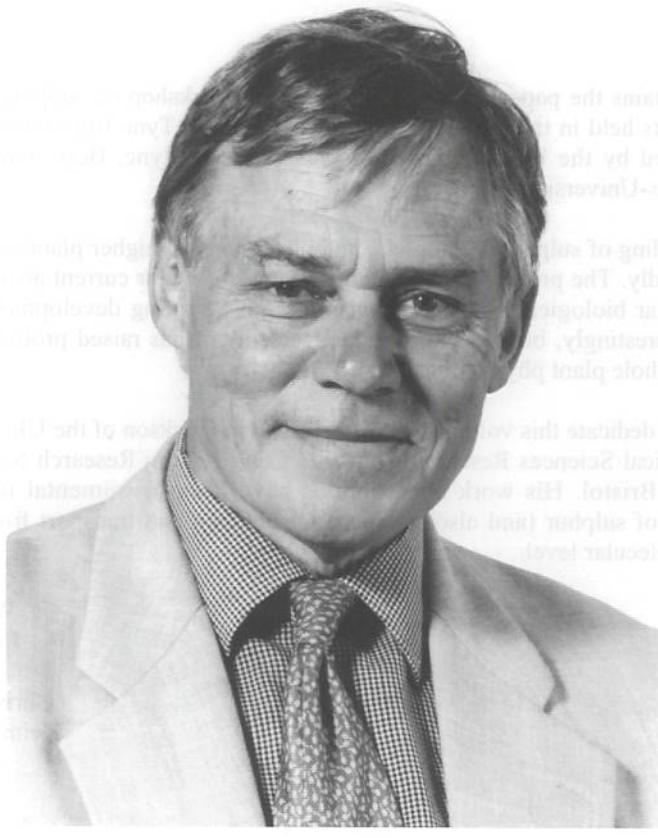
This book contains the papers presented at the third Workshop on Sulphur Metabolism in Higher Plants held in the University of Newcastle upon Tyne from 9th to 13th April, 1996, organised by the Universities of Newcastle upon Tyne, Bern, Groningen, and Albert-Ludwigs-University, Freiburg.

The understanding of sulphur metabolism and physiology in higher plants is progressing ever more rapidly. The present book contains reviews of major current areas of work, of which molecular biological understanding is the most striking development in the last few years. Interestingly, but not entirely unexpectedly, it has raised problems yet to be solved at the whole plant physiological level.

We are glad to dedicate this volume to Professor David Clarkson of the UK Biotechnology and Biological Sciences Research Council's Long Ashton Research Station and the University of Bristol. His work and stimulus have been instrumental in developing understanding of sulphur (and also nitrogen) metabolism and transport from the whole plant to the molecular level.

W. John Cram
Luit J. De Kok
Ineke Stulen
Christian Brunold
Heinz Rennenberg

*editors*



David Clarkson

# FOREWORD

## Sulphate transport and its regulation: a personal view

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### The black box

There was a time when studies of mineral nutrient uptake and of metabolism were pursued by quite different people, often working in different departments and publishing their findings in different journals. As we have come to understand more about the mechanisms which transport ions across membranes such distinctions have become less and less appropriate. At the present time, transport physiologists and metabolism people are brought together by the realisation that the processes which determine the entry of ions into a cell and those which bring about their assimilation, may share common regulatory controls. It is not, then, so strange that a symposium volume about sulphur metabolism should be introduced by some remarks by someone who began to ply his trade in mineral nutrition when, for many practitioners, the process might have been summarised in Fig 1. Of course, there have always been individuals prepared to look into the black box, and it is nice to have this opportunity to pay tribute to a few of them. My own work with sulphate transport began in the early eighties but, by that time, some remarkable papers had appeared which have influenced my own thinking and that of my colleagues ever since. This short piece is, therefore, a very personal, retrospective view of the literature; it is obvious that much significant work will be omitted.

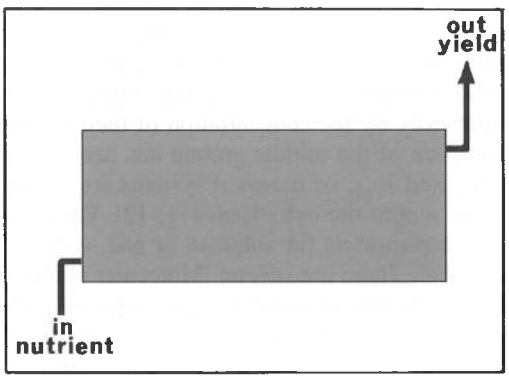


*Fig. 1.* One simplified view of mineral nutrition.

*Sulphur Metabolism in Higher Plants, pp. xiii-xviii*
*Edited by W.J. Cram et al.*

**Entering the black box**

Emanuel Epstein and his colleague Calvin Hagen are generally recognised as the first to point out that ion uptake could be described by the conventions used in enzyme kinetics to relate the velocity of reactions to the concentrations of substrates (8). Thus, ion carriers could be assigned a Michaelis constant ($K_m$), which measured their affinity for the ion carried, and a maximum velocity ($V_{max}$), which measured the capacity of the system to transport ions. This classic work included observations on sulphate transport by barley roots (16). Net uptake at low external concentrations was attributed to a carrier of high affinity for sulphate ($K_m$ 9.5 μM), with a great discrimination against all other anions tested, except for selenate. The $V_{max}$ of this transport system responded positively to the presence of higher concentrations of calcium, which screened out fixed negative charges on the cell surfaces (the so-called Viets effect). It operated at maximal efficiency in mildly acidic solution and depended on aerobic metabolism. These careful observations foreshadowed most of the things we have learned subsequently about sulphate transporters, and pointed to one basis – the sensitivity to selenate, a toxic analogue for sulphate – for screening mutants defective in sulphate transport. The $K_m$ values they obtained correspond closely to those of cloned higher plant sulphate transporters expressed in a yeast mutant, YSD1, which lacks a high affinity transporter of its own (25). Leggett and Epstein (16) also noticed that the uptake over a wide range of external concentrations could be resolved into two phases, the first accounted for by the activity of the high affinity carrier, and a second, evident only at high concentrations, which must have been due to some transport system of lower affinity.

Looking back at the vigorous controversies about Epstein's ideas, in the light of what we have discovered recently, it is hard to understand what the fuss was all about. Broadly, the alternative to the notion that there might be two carrier systems was that a single carrier could exist in a number of discrete states, thus giving rise to multiphasic kinetics. These states would correspond to highly regulated changes in the folding or orientation of the carrier polypeptide so as to influence its binding and transport of the ion. It is significant for my short review that Per Nissen, who has stoutly defended his multiphasic view of the transport process through three decades (see 20 for a comprehensive review), also did some of his earliest work on the uptake of sulphate (19). As so often in biological research, the protagonists in this dispute took exclusive views about the nature of the processes they were interested in. In my opinion, it has never seemed necessary to exclude the idea that the transport activity of either higher or lower affinity systems might be modulated kinetically by the concentration of their substrates on either side of the membrane. The champion of the middle ground has been Tony Glass who has long proposed that both the $K_m$ and $V_{max}$ of transport systems are subject to allosteric regulation by ion concentrations within the cytoplasm (11, 12). On the central question as to whether there are several transporters for sulphate or one, with variable kinetic properties, the answer is uneqivocal. There are several. Molecular cloning has shown that there are versions of sulphate transporters encoded by quite separate genes. As we have found already, some of these can differ substantially in $K_m$, even within the family of high affinity transporters (25, and Hawkesford and Smith, this volume p. 13). In yeast, the gene *SUL1* may be deleted, leaving no possibility that the polypeptide could be expressed in a modified form, but the cells have a residual capability for absorbing sulphate by a low affinity system if sulphate in the external medium is > 2 mM (26). At probable values of the membrane potential in yeast, this low afinity transporter, like the high affinity one, moves sulphate into the cells uphill, thermodynamically. An energised carrier, rather than an ion channel, must be involved, but it must be a different polypeptide.

In this volume, Heinz Rennenberg (p. 59) points out that the entry into the black box considered above, is only one of a series of transports into boxes which are, if anything, even blacker. Transport across the plasma membrane occurs in a number of tissue types within the plant, most of them a great deal less accessible than the plasma membranes of roots; it is likely that versions within the gene family reflect the particular requirements of these processes and the conditions in which they operate (see also Hawkesford and Smith, this volume p. 13).

**Inside the black box**

Ivan Smith began to look, in the 1970s, at what might be at work within the black box. His search for the internal, S-dependent factor, which might play a central role in regulation, strongly influenced our own approach (2) and seemed to point to sulphate itself as the factor (27, 28). His work on cultured tobacco cells showed that full expression of the sulphate transport system was repressed in conditions where the $SO_4^{2-}$ – supply was not limiting growth and the cellular sulphate was abundant. Along with several other groups, we found that less than 10% of the potential capacity was active in such circumstances, but that the system de-repressed quickly when the external $SO_4^{2-}$ – supply was removed. It became clear to us that the sulphate transport system behaved in a relatively straightforward manner in response to sulphate supply. It appeared to be constitutively expressed and did not display the characteristic induction seen for the nitrate transport system (22). The robust responses of this simple system made it, in our view, the best candidate among the major anions for a molecular cloning approach. While this approach has been successful, the identity of the key regulating S compound within cells remains unproven. It is to be hoped that people will not make the mistake of taking too exclusive a view in this matter. Sulphate, cysteine, glutathione and *o*-acetylserine all have their supporters and, at the end of the day we may find that there are several pathways by which such compounds interact with the factors which regulate transcription, or modulate the activity of transporters and enzymes.

I became aware of the work of George Marzluf, on the genetics of sulphate transport and metabolism in *Neurospora*, in the 1980s. His papers made me appreciate how much can be gained by the generation and analysis of mutations (17, 18), but reading them at that time I could only admire the sophistication, seeing little hope of developing such insights in higher plants. A great deal has happened in the intervening decade and a half. Marzluf added molecular cloning to his formidable mutation analyses and a dazzling series of papers identified not only the first eukaryotic gene encoding a high affinity sulphate transporter (14, 15), but revealed the presence of regulatory genes, the products of which coordinate the expression of a number of enzymes of sulphur metabolism, according to the cell sulphur-status (10). The *cys-3+* gene product is a DNA-binding protein which acts in a positive way to turn on the expression of genes for sulphate permeases and for enzymes of sulphur catabolism. These genes share a common sequence in their promoters, which binds the CYS3 protein. Working with the promoter of the *cys-14* sulphate permease gene, DNA footprint analysis has shown that there are three motifs which bind the CYS3 protein (13). There are also two regulatory genes which act in a negative fashion, one of which, *scon-1*, appears to strongly inhibit the synthesis of the *cys-3* mRNA; all three of these regulatory genes respond to the S-status of the cells. It remains to be seen whether or not higher plants possess the same kind of global regulatory circuits as have been described in *Neurospora*, and in yeast (see the contribution by Surdin-Kerjan on p. 27 of this volume), but without doubt these models indicate the way

we should go. The laborious business of promoter analysis of the various genes has not really begun with higher plant models, but the paper of Kanaan and Marzluf (13) indicates that the effort will be worthwhile.

Perhaps it should not have seemed so surpising, but there was certainly excitement generated by the observations that there was some kind of co-ordinated regulation of N and S metabolism. A number of reports appeared in the mid-70s showing how nitrogen utilisation by plants became very inefficient when S was inadequate (9, 21) but fewer people appeared interested in the reciprocal effect of N nutrition on S metabolism. Reuveny and Filner (23) showed that ATP sulphurylase formation depended on the level of the nitrogen supply, and later Reuveny expanded this idea into a more general proposition that end products of N and S metabolism affected the expression of enzymes of the assimilatory pathways in a complementary way (24; see also 28). Our own work suggested that there might be, in addition, down-regulation of the plasma membrane transport of $NO_3^-$ or $SO_4^{2-}$ in the early stages of their respective deficiencies (3). The validity of adding the transport steps to the more thoroughly researched metabolic interactions between N and S, is supported by the rapid de-repression of $SO_4^{2-}$ influx by *O*-acetylserine in S-sufficient roots and cells where transporter activity is usually heavily repressed.

It is risky to attribute "purpose" to natural phenomena, it may even be a delusion to think in such terms. It is, however, intriguing to speculate why it is that $SO_4^{2-}$ uptake is so tightly regulated. Excess $SO_4^{2-}$ intake could be dealt with either by vacuolar sequestration (sometimes to very high concentrations; see Ernst, this volume p. 131), or by accumulation of glutathione. Is there an imperative to keep cytoplasmic $SO_4^{2-}$ levels relatively low, as suggested by Cram (7) in the first of these symposia? We are no nearer answering this question, because we still lack any method which can give direct information of cytoplasmic sulphate pool-size.

**Boxes within boxes**

This symposium volume begins with a contribution from Enrico Martinoia on transport across the tonoplast and several later papers highlight the importance of sulphate transport in and out of vacuoles. Cram (5, 6) pointed out that the rate constant for sulphate turnover in the vacuole can be slower than that for tunover in the cytoplasm. This probably contributes to the well-recognised symptoms of sulphur deficiency, which appear in young leaves while old leaves contain a substantial amount of stored sulphate. In *Macroptilium atropurpureum*, the rate constants in root vacuoles are appreciable greater than in leaves (1). We know very little about the molecular nature of the transport mechanisms for entry into the vacuole or for efflux. This represents a major challenge; it may prove much more difficult to devise workable screening protocols which will reveal mutants with altered tonoplast transport properties. If processes in the cytoplasm are, indeed, sensitive to excess sulphate, one might search for mutants showing unusual sensitivity in their growth and metabolism to external sulphate; this is, however, a negative screen and not easy to manage. There has been no further progress in identifying the putative sulphate transport mechanism in the chloroplast since the last symposium (4); in root plastids from pea, however, there appeared to be no evidence for a transporter, sulphate diffusing readily across the envelope (Bowsher and Clarkson, unpublished results).

## A personal footnote

I think that I, and my contemporaries, have been very fortunate that our professional lives have developed in the mid to latter part of this century. Until the most recent decade, we enjoyed stability and regular funding which is now, sadly, denied to those who follow us. This gave us the opportunity to agonise over intractable problems in our science rather than preside over the management of a permanent crisis. This experience made us quick to recognise how advances of molecular biology could be harnessed. Lateral thinking is often the product of long experience. This is one of the principal sources of regret about the short-term nature of funding. New initiatives follow quickly on the heels of earlier ones; the flickering funding spotlight dances nervously over the great gathering of needy supplicants clamouring at the gate of the research councils, picking out a group here and there for a short period and then swinging to focus elsewhere. More than anything else, I feel outraged about the way in which we now treat our younger colleagues. I am amazed and humbled by their resilience and dedication in such an insecure system; it is wrong that their own creative development should be subdued so that baleful milestones and deliverables can be checked off the lists, required in many modern proposals, by the agreed deadlines.

I am deeply grateful to the editors for dedicating this symposium volume to me. As I have suggested above, I feel that I have had a fortunate life and this unexpected honour confirms that feeling. The volume itself is an interesting one, especially if it is compared with those produced from earlier symposia. The pace of new discoveries quickens; it is clear that molecular biology does more than describe familiar systems and problems in a new way, it brings insights which could only be dreamt of when I started in this business.

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# TRANSPORT OF SULPHATE AND REDUCED SULPHUR COMPOUNDS AT THE TONOPLAST MEMBRANE

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## Introduction

The vacuole is the largest compartment of a mature plant cell and may occupy more than 80% of the total cell volume. In such mature cells, the cytosol is visible only as a thin layer which is separated from the cell wall by the plasma membrane and from the vacuolar sap (cell sap) by the vacuolar membrane (tonoplast). The constituents of the cell sap are water and mainly inorganic salts. Therefore the vacuole allows the plant to reach a large size and a large surface area while spending only a minimum of energy on the energy-consuming synthesis of metabolites by accumulating salts from the environment which osmotically drive further water uptake.

The requirement of cytosolic ion homeostasis is also served by the vacuole acting as an internal reservoir of metabolites and nutrients (3, 20, 28, 33, 34). Investigations of vacuolar constituents show that these may vary between and within plant species, and depending on the environmental conditions. Import and export of metabolites and nutrients across the tonoplast have, therefore, to be strictly controlled to permit an optimal functioning of the metabolically active compartments. Regulatory mechanisms must exist at the cytoplasmic face of the tonoplast which is an entity of the cytoplasm.

## Vacuolar localization of sulphur containing substances

As shown in Table 1, changing the nutritional conditions of barley plants by the stepwise addition of nitrate, chloride or sulphate to a hydroponic medium results in an increased cellular concentration of the respective ion. Since the extravacuolar concentration of the different ions remains relatively constant, the highly increased ion content reflects an increased concentration within the vacuole. Similar observations were reported when ion concentrations in freshly isolated intact chloroplasts from spinach plants treated with different ion concentrations were determined (20). In this case, the chloroplastic concentrations remained relatively constant compared to those in the leaf extracts. Increase of chloroplastic ion concentrations would affect metabolism. Sulphate, for example, has been shown to be a competitive inhibitor of chloroplast ribulose-1,5-bisphosphate carboxylase (18) and an inhibitor of photophosphorylation as well (17).

*Sulphur Metabolism in Higher Plants, pp. 1-11*
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*Table 1.* Distribution of various anions in control and salt stressed plants. Data from Kaiser et al. (1989) and Martinoia et al. (1987)

| Treatment | anion | Concentration of the respective anion [mM] in protoplasts | in vacuoles | in extravacuolar |
|---|---|---|---|---|
| control | $Cl^-$ | 56 | 56 | 56 |
| + 250 mM NaCl | | 153 | 180 | 47 |
| control | $NO_3^-$ | 35 | 43 | 4 |
| + 250 mM $NaNO_3$ | | 156 | 195 | 7 |
| control | $SO_4^{2-}$ | 6 | 5 | 7 |
| + 250 mM $Na_2SO_4$ | | 71 | 75 | 11 |

Sulphate is usually the major sulphur containing compound detected within the vacuole. Some organic sulphur compounds such as methionine, glutathione and conjugates of glutathione have also been found (8, 41, 52), whereas cysteine is usually not detectable in vacuoles. The vacuolar proportion of methionine may vary with the total cellular methionine content as has been shown for other amino acids (29). Glutathione concentrations in vacuoles are generally very low (8, 41). Interestingly the ratio of GSH to GSSG is lower in the vacuole than in the cytosol. Sulphur-containing secondary plant products, such as glucosinolates, which are mainly found in the family Brassicaceae where they are involved in the protection of plants against herbivores, are localized within the vacuole (14).

## Transport of inorganic sulphur across the tonoplast

Two excellent reviews related to sulphate transport, including vacuolar transport, have been contributed by Cram (7) and Clarkson *et al.* (5) to the proceedings of previous Sulphur Workshops. As only little additional information has become available during recent years we will only briefly refer to vacuolar sulphate transport.

It has been reported that plants subjected to S-stress retain $SO_4^{2-}$ to a greater extent in the leaves than in the roots (6). Furthermore, in *Macroptilium atropurpureum* newly emerging leaves showed symptoms of S-stress although mature leaves were found to contain appreciable quantities of sulphate. Tracer exchange experiments for the vacuole and the cytosol showed that the rate constant for the exchange of vacuolar ($k_v$) $SO_4^{2-}$ is low compared to that of the cytosol ($k_c$), indicating that under $SO_4^{2-}$ starvation the cytosolic pool is depleted faster than the vacuolar one. Recently, Bell et al. (2) investigated the rate constants in roots and leaves of *Macroptilium atropurpureum*. In leaves they found two different values for $k_v$ ($3.54 \times 10^{-2}$ $h^{-1}$ and $3.33 \times 10^{-4}$ $h^{-1}$). The rate constant of the faster exchanging compartment was assumed to reflect the exchange in the epidermis, the slower one that in the mesophyll. A value similar to the faster component was found for the turnover in root vacuoles ($3.78 \times 10^{-2}$ $h^{-1}$). The rate constants for the cytoplasm were all very similar and at least two orders of magnitude greater (2.46 – 2.58 $h^{-1}$).

Efflux analysis of anions from isolated, intact vacuoles indicate that $SO_4^{2-}$ and $HPO_4^{2-}$ cross the tonoplast at much lower rates than either $NO_3^-$ or $Cl^-$ and are therefore in agreement with tracer experiments on whole plants (19). The same sequence of relative permeabilities is observed when tonoplast vesicles are used. However, in this case the orientation

of the membrane is not defined (e.g. 4). Efflux of $SO_4^{2-}$ could be increased by the addition of gramicidin, but not of nigericin, in the presence of potassium, indicating that the electrical potential difference ($\Delta\psi$) is the driving component for tonoplast influx. However, the membrane potential across the tonoplast has been reported to vary between 10 and 30 mV (vacuole positive) and it is unlikely that negative membrane potentials occur *in vivo*.

To our knowledge, transport of sulphate across the tonoplast has been investigated in detail only in barley mesophyll vacuoles (19). Uptake is stimulated by MgATP, resulting in a net accumulation inside the vacuole. In the presence of inhibitors of the tonoplast $H^+$-ATPase (42), namely DCCD or DES, $SO_4^{2-}$ -uptake was reduced to the non-energized level, indicating that energization was due to the activity of the vacuolar proton pump. Concentration-dependent sulphate transport has been reported to be either saturable with a $K_m$ value of 3.8 mM (8) and 2.2 mM (Hörtensteiner and Martinoia, unpublished results) or biphasic with a saturable (Km 0.5 mM) and a linear component (19). Due to the fact that transport of different anions, such as nitrate, chloride, sulphate or malate, utilizes $\Delta\psi$ as energy source, it is difficult to investigate whether a single or several carriers are involved in the transfer of different anions, since inhibition of the uptake of one anion by another could also result from competition for energy. However, by the use of different protein modifying agents it was established that chloride and malate are transported by distinct carriers (32). Table 2 shows that malate and sulphate also cross the tonoplast by distinct carriers. Sulphate transport is not affected by 1, 2, 3-benzenetricarboxylic acid but is very strongly inhibited by $CrO_4^{2-}$, while malate transport is inhibited by the tricarboxylate but not by chromate. The situation is less clear for chloride and sulphate, however, chloride uptake being inhibited to a lesser extent by chromate than is the uptake of sulphate. The $K_i$ values for chromate inhibition were estimated to be between 50 and 80 µM for sulphate and 1 to 2 mM for chloride. Furthermore, sulphate uptake is inhibited by phenylglyoxal which has no effect on chloride transport (see also 32). These results indicate that chloride and sulphate are transported by two different carriers. However, it cannot be excluded that an additional common transport system exists reflected by the linear component which has been observed in both uptake systems (19, 30).

During sulphur or phosphate starvation, uptake of sulphate and phosphate at the plasma membrane is increased (15). For barley mesophyll vacuoles it has been shown that phosphate starvation also leads to an increased vacuolar uptake activity (35). It would, therefore, be interesting to investigate whether $SO_4^{2-}$ uptake activity is also enhanced in vacuoles of sulphur starved plants.

Due to air pollution, large amounts of $SO_2$ may be taken up by leaves. $SO_2$ dissolves in cell water to give $HSO_3^- + H^+$ and $SO_3^{2-} + 2H^+$, which are acids and are therefore excluded from an acidic compartment like the vacuole (39). The permeability of the tonoplast for this compound is comparable to that of the plasma membrane (39). Due to the rapid

*Table 2.* Inhibition of the transport of $SO_4^{2-}$, malate and $Cl^-$ by competitive inhibitors and protein modifying agents. Substrate concentrations were 0.5 mM for sulphate and malate and 1.2 mM for chloride. Values shown are means of two experiments, each with five replicates

| Treatment | Transport (% of control) | | |
|---|---|---|---|
| | $SO_4^{2-}$ | malate | $Cl^-$ |
| Benzenetricarboxylic acid (5mM) | 82 | 5 | 92 |
| $CrO_4^{2-}$ (2mM) | 0 | 89 | 53 |
| Phenylglyoxal (5mM) | 38 | 41 | 90 |

oxidation of $SO_2$ to $SO_4^{2-}$ within the cell or, alternatively, its detoxification by reduction (43), it is not likely that $HSO_3^-$ or $SO_3^{2-}$ are accumulated within the vacuole *in vivo*. The cell has therefore to cope with $SO_4^{2-}$ and $H^+$. Calculations show, that the vacuolar transport capacity for $SO_4^{2-}$ is more than sufficient to transport all sulphate produced by oxidation within the cell, even in air heavily polluted with $SO_2$. Furthermore, the leaf cell also has to cope with the resulting acidification which may be more dangerous.

**Transport of methionine**

In barley vacuoles, transfer of amino acids has been shown to be catalyzed by a general transport system (11, 31). As for other amino acids, methionine uptake into vacuoles is modulated, but not energized, by ATP, since uptake is stimulated by Mg-free ATP or by the non-hydrolysable ATP analogue AMPPNP (adenylyl imidodiphosphate). Nucleotides other than ATP and its analogues failed to stimulate the transport of amino acids. The rate of amino acid uptake as a function of ATP concentration exhibits a sigmoidal curve. ATP is ineffective at concentrations below 1 mM and maximal activation is found between 3 and 5 mM. Phenylalanine, leucine and methionine inhibit the ATP-stimulated uptake of other amino acids almost completely at a concentration of 4 mM. The ineffectiveness of D-phenylalanine, phenylethylamine and phenylpropaneoic acid indicates a specificity for L-amino acids in inhibiting the ATP-stimulated amino acid uptake. Various amino acids may respond differentially to ATP: uptake of methionine as well as of glutamine and leucine is stimulated both by free ATP and MgATP (9), whereas the uptake of other amino acids such as arginine, glycine or aspartate is stimulated only by free ATP (9).

Efflux of methionine and other amino acids from isolated vacuoles exhibits very similar properties i.e. stimulation by ATP and inhibition by hydrophobic amino acids such as phenylalanine or methionine. It is, therefore, likely that amino acid in- and efflux are mediated by the same transport system (10). As pointed out by Dietz et al. (8), there is a good correlation between the uptake of a given amino acid in the absence of ATP and its hydropathy index (Table 3). The higher uptake activity for arginine than expected can be explained by a vacuolar transport system which specifically recognizes positively charged amino acids. In the presence of ATP, transport rates for different amino acids are highest for arginine while those of the negatively charged aspartic acid remain low. Small neutral amino acids cross the membrane faster than larger ones (Table 3). It is not yet clear, whether inhibition of amino acid transport by hydrophobic amino acids is due to a competitive inhibition or whether another part of the permease is able to bind hydrophobic amino acids and is responsible for the modulation of the transporter. An open question is the role and the mode of action of this transporter *in vivo*, at least for amino acids which cross the membrane only in response to free ATP since the concentration required to activate the transporter is much higher than that reported in the cytosol (31). Furthermore, at their known cytosolic concentrations hydrophobic amino acids would be expected to inhibit this transporter almost completely.

*Table 3.* Velocities of uptake of different amino acids into isolated barley vacuoles. Values from Dietz et al. (1990), Martinoia et al. (1991) and Dietz et al. (1992)

| Amino acid | Uptake rates (nmol × $10^{-7}$ vacuoles × $min^{-1}$) -ATP | + ATP | hydrophobicity |
|---|---|---|---|
| arginine | 0.48 | 3.92 | 12.3 |
| aspartic acid | 0.06 | 0.26 | 9.2 |
| glutamine | 0.26 | 2.10 | 4.1 |
| methionine | 0.74 | 1.50 | 3.4 |
| cysteine | 0.57 | 0.56 | 2.0 |

## Transport of glutathione conjugates

Detoxification and elimination of potentially toxic compounds such as microbial toxins or agrochemicals (xenobiotics) taken up by the plant are a prerequisite for its survival. Metabolism and detoxification of xenobiotics are remarkably similar in plants and can generally be divided in two or three phases (16, 21, 45), depending on the nature of the compound. In the first phase, generally mediated by cytochrome P-450-dependent monooxygenases, a foreign compound may be oxidized, reduced or hydrolyzed to either introduce or reveal a functional group. In the second phase, the activated or unmodified xenobiotic is conjugated to a hydrophilic compound such as glucose, glutathione, glucuronate (only known in animals), or malonate by the respective transferases. Finally, conjugated xenobiotics, now being more hydrophilic than the respective parent compound, are excreted into the vacuole.

The large number of xenobiotics with diverse chemical structures provokes the question how transporters of the vacuolar membrane are able to recognize the different substrates. ATP is required for vacuolar transport of glutathione conjugates, such as NEM-glutathione, metolachlor-glutathione, dinitrobenzene (DNB)-glutathione and oxidized glutathione (a special case of a glutathione conjugate) (23, 27). Glutamate and cysteine, both components of glutathione, as well as reduced glutathione or cysteine conjugates, did not interfere with NEM-glutathione uptake, whereas glutathione conjugates such as those of DNB, symetrin and metolachlor were all inhibitory. Long chain, linear and hydrophobic glutathione conjugates such as decyl-glutathione most strongly interfere with the uptake of other glutathione conjugates (Tommasini and Martinoia, unpublished). Competitive inhibition of the uptake of GSSG and NEM-glutathione, respectively, by metolachlor-glutathione had identical $K_i$ values. Therefore it was postulated that a single transporter is responsible for the transport of the different glutathione conjugates across the tonoplast (50).

Surprisingly, uptake of either NEM-glutathione or metolachlor-glutathione was not inhibited by bafilomycin, a very specific inhibitor of the tonoplast $H^1$-ATPase. Dissipation of the transtonoplast $\Delta pH$ (by $NH_4^+$ or FCCP) or $\Delta\psi$ (by valinomycin) had very little or no effect on glutathione-conjugate uptake. In contrast, vanadate, known to inhibit ATPases which form phosphorylated intermediates during ATP hydrolysis (plasma membrane $H^+$-ATPase, $Ca^{2+}$-ATPase but not the vacuolar $H^+$-ATPase) strongly inhibited the uptake of glutathione conjugates. These results indicate that glutathione conjugate uptake is driven neither by the vacuolar $H^+$-ATPase nor by a pre-existing proton gradient. Indeed, a five to six fold accumulation of metolachlor-glutathione within isolated vacuoles relative to the exogenous concentration was detected even in vacuoles which were incubated in the presence of bafilomycin and $NH_4^+$. Furthermore, the $K_m$ for MgATP stimulation of glutathione conjugate transport was significantly lower than the values reported for $H^+$ transfer. Combined, these results indicate that glutathione conjugate transport is mediated by a spe-

cific ATPase. The activity of this ATPase is strikingly similar to the glutathione conjugate transport activities of animal cells residing in their plasma membrane (36) and in the vacuolar membrane of fungi (46). A notable difference between animals and plants is the fact that only the plant enzyme can be inhibited by vinblastine and verapamil (23) (see also Table 4).

$\Delta G^o$ of ATP hydrolysis calculated on the basis of the known cytosolic concentrations of ATP, ADP and Pi, can drive a much higher vacuolar accumulation by a directly energized glutathione-conjugate pump compared to secondary energized transport mechanisms (21). Furthermore, degradation within the vacuole of a GS-conjugate to the respective cysteine conjugate is often observed for herbicides and may represent an additional trapping mechanism. The first enzyme for this degradation, a carboxypeptidase yielding the $\gamma$-glutamyl-cysteine conjugate has recently been localized within the vacuole (52, Kreuz and Martinoia, unpublished). It must be assumed that efficient removal of glutathione conjugates from the cytosol is a necessity for plants, since glutathione conjugates may in fact exert biological activities dangerous for plant cells. GS-conjugates of herbicides might likewise act as inhibitors of glutathione transferases and thereby impair the capacity for efficient metabolism of these xenobiotics (22).

Plants may tolerate higher concentrations of herbicides when treated with so called safeners. Safeners are a group of structurally diverse chemicals which often induce a very specific reaction of a crop towards specific herbicides. Increased activities of glutathione *S*-transferases, cytochrome P-450-dependent monooxygenases and glucosyl transferases as well as increased GSH contents have been observed in response to safener treatment (12). It was therefore interesting to investigate whether transport activities of glutathione conjugates are also increased under these conditions. Indeed, treatment of barley plants with cloquintocet-mexyl, a safener specific for small grasses, strongly enhanced the rate

*Table 4.* Characteristics of the vacuolar glutathione conjugate transporter.
a – Martinoia et al., (1993); b – Li et al. (1995); c – Tommasini et al. (1993); d – Hörtensteiner et al. (1993) and e – Tommasini and Martinoia, unpublished

| Property | values/comments |
|---|---|
| $K_m$ (substrates) | 500 µM (NEM-GS)[a]; 50 µM (metolachlor-GS)[a]; 81 µM (DNB-GS)[b]; 500 µM (GSSG)[c] |
| $K_m$ (ATP) | 90 – 100 µM[a]; 51 µM[b] |
| Stimulation by other nucleotides | GTP (83% at 3 mM)[a,d]; UTP (51% at 3 mM))[a,d]; ADP (0%)[d]; AMPPNP (0%)[a,b]; PPi (O%)[b,d] |
| Not inhibited by(potential substrates) | glu[a], cys[a], gly[b], cysteine-NEM[a], GSH[a,b] |
| Inhibited by (potential substrates) | all glutathione *S*-conjugates tested so far, including GSSG |
| Not inhibited by | FCCP[b], CCCP[d], $NH_4Cl$[a,b,d], $NaN_3$[a,b], valinomycin[a,b], bafilomycin[a], $NaNO_3$[b] |
| Inhibited by | vanadate ($I_{50}$ 7.5 µM[b], 60 µM[a]), oligomycin (87% at 20 µg/ml)[d]; vinblastine ($I_{50}$ 26 µM)[b]; verapamil ($I_{50}$ 39 µM)[b] |
| GS-conjugate pump present in | barley leaf[a], *Arabidopsis* leaf and root[b], maize root[b], beet storage root[b], mungbean hypocotyl[b], carrot taproot[e], *Hevea* lutoids[e] |

of glutathione conjugate uptake into barley vacuoles (13). This effect was not observed with safeners used for maize protection but which are inactive in barley. The activity of the glutathione conjugate pump can also be enhanced by substrates of GSTs such as 1-chloro-2,4-dinitrobenzene, as shown for mung bean (24). The fact that the activity of the glutathione conjugate pump is increased simultaneously with other enzymes involved in the detoxification of xenobiotics or in the presence of glutathione conjugates generated within the cell (DNB-GS) supports the hypothesis that elimination of the potentially toxic glutathione conjugates is an integral part of the detoxification mechanism.

We have not been able to identify and purify the glutathione conjugate transporter using biochemical methods. Glutathione-conjugate-dependent ATP-hydrolysis was too low to follow the activity of the transporter during a purification procedure and the application of radiolabelled photoreactive glutathione conjugates resulted in protein labelling of low specificity.

However, the characteristics of the transport system let us suppose that the glutathione conjugate pump could be a member of the ABC transporters (1). Monoclonal antibodies directed against the conserved ATP binding region of an ABC transporter actually inhibitied DNB-glutathione transport (Table 5). In contrast, antibodies directed against a non-conserved region produced no effect.

While in yeast a large number of genes coding for such transporters are known, only a few substrates transported by the respective proteins have been identified. As in plants, the glutathione conjugate transporter of yeast is localized on the vacuolar membrane (25, 49). Testing available yeast strains deleted in the gene coding for individual ABC transporters for their capacity to transport glutathione conjugates showed that a cadmium-sensitive yeast strain with a deletion in the *YCF 1* gene (48) had a strongly reduced glutathione conjugate transport activity (10 to 20% of the control value; 25, 49), indicating that the gene product of *YCF 1* is a glutathione conjugate transporter. YCF 1 is highly homologous to another member of the ABC family, namely the human MRP (multi drug resistance associated protein). Expression of *MRP* in human cancer cells resulted in an increased transport activity for glutathione conjugates (36). Very recently it was shown that the heterologous expression of *MRP* in the *YCF1* mutant restores the glutathione transport activity. Furthermore, *YCF1* mutants expressing MRP were again tolerant to cadmium (49). However, it could not be demonstrated that the cadmium-glutathione complex is transported across the tonoplast. So while YCF1 appears to be a glutathione conjugate transporter it may not also be a Cd-glutathione transporter. The molecular mechanism of for cadmium tolerance therefore still remains to be identified.

Since glutathione conjugates with naturally occurring substrates have not been demonstrated unequivocally, the physiological function of the glutathione conjugate ATPase is still unknown. New light on this question was recently shed by Marrs et al. (26). They showed that in maize the gene product of *Bronze 2* which catalyzes the last step in anthocyanidine biosynthesis is a GST. Transfer of this flavonoid into the vacuole occurred only when *Bronze 2* was expressed, and two-dimensional TLC of plant extracts,

*Table 5.* Inhibition of [$^{14}$C]-GS-metolachlor uptake in isolated barley vacuoles by monoclonal antibodies directed against the ABC motif and a non-conserved region of MRP (36). Antibodies were a gift of Dr. Zaman, Netherland Cancer Institute, Amsterdam, The Netherlands

| Treatment | activity (% of control) |
|---|---|
| AK 6 MRP (directed against the ABC domain) | 59 ± 4 |
| AK MRP 1 (directed against a non-conserved region) | 91 ± 6 |

showing co-migration of anthocyanin and radiolabelled glutathione, suggests that in maize anthocyanin is transferred into the vacuole as its glutathione conjugate. A further and more general role for the glutathione conjugate pump may be the transfer of oxidized glutathione into the vacuole during oxidative stress (21, 50).

## Transport of phytochelatins

Detoxification of heavy metals, mainly cadmium, occurs via the production of a cadmium-phytochelatin ($Cd^{2+}$-$(\gamma$-glu-cys$)_n$-gly) complex (40, 47). Both phytochelatins and cadmium have been localized mainly within the vacuole (51). A Cd-sensitive mutant of *Schizosaccharomyces pombe*, capable of synthesizing phytochelatins but unable to form large amounts of cadmium phytochelatin sulphide complexes was shown to be mutated in an ATP-binding casette type transport protein with a molecular mass of 90.5 kD ("HMT1" for heavy metal tolerance 1) (37). Despite the fact that both HMT1 and YCF1 confer heavy metal tolerance, these two ABC transporters are not closely related. As mentioned above *YCF1* shows highest homology to *MRP*, whereas *HMT1* is related more closly to *MDR 1* and *Ste6*. The gene products of both *HMT1* and *YCF1* reside in the vacuolar membrane. Phytochelatins were taken up by vacuoles isolated from the wild type or from HMT1 overexpressing cells, but not from mutants deleted in *hmt1*. Cadmium-phytochelatin as well as apo-phytochelatin are transported across the vacuolar membrane at similar rates (38). However, transport of the high molecular weight phytochelatin-cadmium-$S^{2-}$ complex was much slower and it could not be excluded that the transport observed was due to degradation of the complex. As for glutathione conjugates, uptake was directly stimulated by ATP which could be substituted by GTP and UTP. In contrast, glutathione conjugate transport was not affected in the mutant, indicating that two different transport systems are involved in the transport of glutathione conjugates and phytochelatins.

Uptake of phytochelatin has also been demonstrated in plant vacuoles (44, Tommasini, Grill, Martinoia, unpublished) and been shown to have properties very similar to those described in yeast. Since a $Cd^{2+}/H^+$ antiport system is present in the tonoplast of plants and yeasts the stochiometry of phytochelatin to cadmium uptake could not be determined. However, we observed that both the phytochelatin-cadmium complex as well as apophytochelatin were taken up by barley vacuoles (Tommasini, Grill, Martinoia, unpublished). In contrast to yeast, the particular gene has yet not been identified in plants.

## Conclusions

Several vacuolar membrane transport systems for inorganic and organic sulphur compounds have been described in recent years. A still open question is how sulphur-containing secondary plant products, such as glucosinolates, cross the tonoplast. Exploiting homologies with yeast and animal systems it should be possible in the next few years to identify the genes responsible for the transfer of glutathione conjugates and phytochelatin in plants. The identification of the other transport sytems for sulphur-containing substances will probably be more complex since no simple strategy can be envisaged and furthermore since we do not know whether a homology between the vacuolar and the already identified plasma membrane sulphate transporter can be expected.

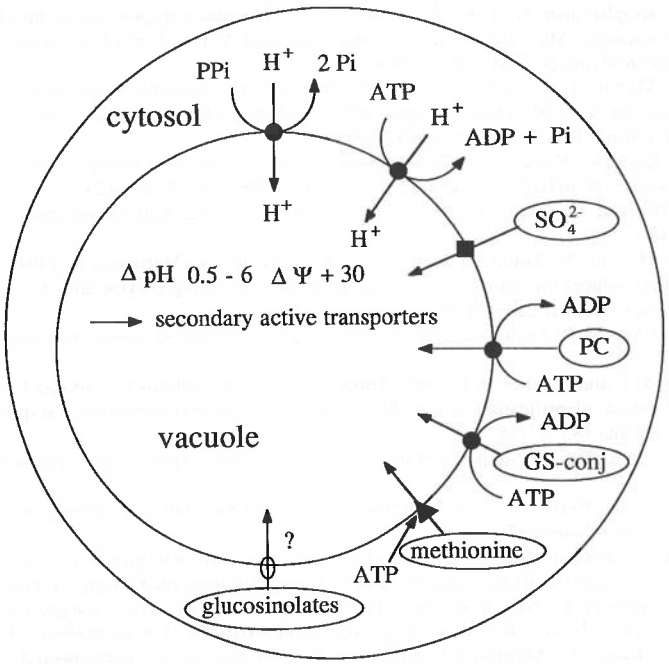


*Fig. 1.* Vacuolar transport systems for sulphur-containing substances. For the description of the individual transport systems see text.

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# MOLECULAR BIOLOGY OF HIGHER PLANT SULPHATE TRANSPORTERS

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## Abstract

Phenotypic complementation of a yeast mutant, YSD1, deficient in sulphate uptake, has enabled the cloning of yeast and plant genes for plasma membrane sulphate transporters. These genes encode polypeptides of between 644 and 859 amino acids in length ($M_r$s 69800 to 96000). When expressed in the yeast mutant, they restore the capacity for sulphate uptake, and may be sub-divided into two categories distinguished by their affinities for sulphate (6.9-11.2 μM and 99.2 μM). Amino acid sequence analysis indicates that there is a family of related sulphate transporters with examples found in bacteria, yeast, fungi and animals as well as in plants. This family bears no resemblance, at the amino acid primary sequence level, to any other known group of transporter proteins. A structural model for the sulphate transporter with 12 membrane spanning domains is presented. Expression studies show that the higher affinity group are expressed solely in the root tissue and that an increased abundance occurs during conditions of sulphur limitation. A positive regulation of expression, by exogenously applied O-acetyl serine, overcomes the observed repression under sulphur-adequate conditions, concomitant with an increase in internal cysteine and glutathione content.

## Introduction

In the previous volume of this series (5), it was speculated that yeast mutants would facilitate the cloning of the structural genes involved in sulphate transport. Considerable effort had been expended previously on diverse approaches toward this goal. However, the strategy which has brought success was, as speculated, heterologous expression of plant genes in a yeast mutant. The cloning of plant genes for sulphate transport coincided with the isolation of several other eukaryotic sulphate transporter genes to add to what was, in 1993, the sole cloned member of this family, the *Neurospora crassa cys-14* gene (18). The occurrence of members from a range of organisms and the comparison of functional characteristics and derived amino acid sequence, helps in elucidating important structural and functional features. From a mechanistic point of view, structure-function studies of sulphate transporters are likely to be particularly illuminating. This is because the sulphate transporters form a unique group of proteins, seemingly unrelated to any of the other wide range of cloned integral membrane proteins, many of which fall into clear subgroups of the major facilitator superfamily of transporters (10, 25). The availability of clones of plant sulphate transporters now permits the study of a key component of the sulphur assimilatory pathway in plants, the elucidation of the coordinated regulation of sulphate transport and assimilation, and presents opportunities to manipulate sulphur metabolism through engineering expression or function.

*Sulphur Metabolism in Higher Plants, pp. 13-25*
*Edited by W.J. Cram et al.*

**Cloning of sulphate transporter genes in yeast mutants**

The concepts underlying the use of yeast mutants to isolate plant transporter genes has been reviewed recently (8). The key component of this strategy is the availability of a specific yeast mutant, which, in the case of sulphate transport, would be a mutant lacking the capacity to take up sulphate. Utilizing a protocol similar to that described by Breton and Surdin-Kerjan in 1977 (2), we selected such a mutant (32); Fig. 1 summarizes the strategy used. Yeast strain InvSc1 (Invitrogen), containing a uracil auxotrophic marker, was mutagenised and mutants were selected for tolerance to the two toxic analogues of sulphate, selenate and chromate. This was done by growing the mutagenised populations on sulphate-free medium containing 75 μM selenate plus 150 μM chromate and homocysteine thiolactone as a S-source. A number of possible mutants from different complementation groups could arise from this strategy. In order to distinguish transporter mutations from other classes, such as those in ATP sulphurylase or global control genes, only mutants with normal levels of expression of ATP sulphurylase were selected. Assays of their capacity to take up sulphate confirmed serious impairments to the sulphate transport function in these mutants. One of these mutants, CRS149, was selected for complementation studies and was transformed with a yeast cDNA library constructed in the yeast shuttle vector pYES2. This vector places the inserted cDNA under the control of the *GAL1* promoter which allows control of expression by growth in the presence of either glucose (expression repressed) or galactose (expression induced).

A 2775 bp cDNA that complemented the mutation in strain CRS149 was isolated and sequenced. This cDNA contained an open reading frame which encoded a 859 amino acid polypeptide which had substantial homology to the sulphate transporter from *N. crassa* (18). This gene was designated *SUL1* and its gene product SUL1. The yeast genomic sequencing project also identified a genomic sequence for this gene on chromosome II (ORF YBR294w, accession no Z36163) by analogy with the *N. crassa cys-14* sequence. A stable deletion mutant, YSD1, was constructed by deleting a 1096 bp *Bcl*I/*Bgl*II fragment from the centre of the *SUL1* coding region, subcloning this fragment into the yeast integrating plasmid yIp5, and using a two-step homologous recombination procedure to replace the *SUL1* gene in strain InvSc1 with the deletion fragment. The strain YSD1 does not take up sulphate from low external sulphate concentrations and grows only slowly on high (5-25 mM) sulphate compared to the wild-type which grows adequately on 50 μM sulphate. YSD1 is routinely maintained on homocysteine thiolactone as a S-source, a reduced S-compound which does not repress the methionine biosynthesis pathway in yeast. Transformation of YSD1 with pYES2 containing *SUL1* and expression facilitated by growth on galactose restored the capacity of this mutant to take up sulphate from low external concentrations and enabled the $K_m$ for sulphate for the SUL1 transporter to be determined as 7.5μM. Subsequent work from the laboratory of Surdin-Kerjan (this volume) has shown that there is a second sulphate transporter gene in yeast, with similar functional characteristics, but which is differentially expressed and was not observed under our conditions and in our media.

Two plant cDNA libraries were constructed in a modified yeast shuttle vector pYES2s using mRNA isolated from roots of sulphur-stressed *Stylosanthes hamata* cv Verano (a tropical forage legume) and roots of sulphur-starved *Hordeum vulgare* (barley). These libraries were introduced into yeast strain YSD1 and transformants that complemented the deletion mutation in the SUL1 gene of this strain were selected. Three different cDNA clones were isolated from the *Stylosanthes* library (31), whilst only one cDNA was identified from the barley library (Smith *et al.*, unpublished and this review). The open reading frames encoded in these cDNAs were designated *shst1*, *shst2*, *shst3*

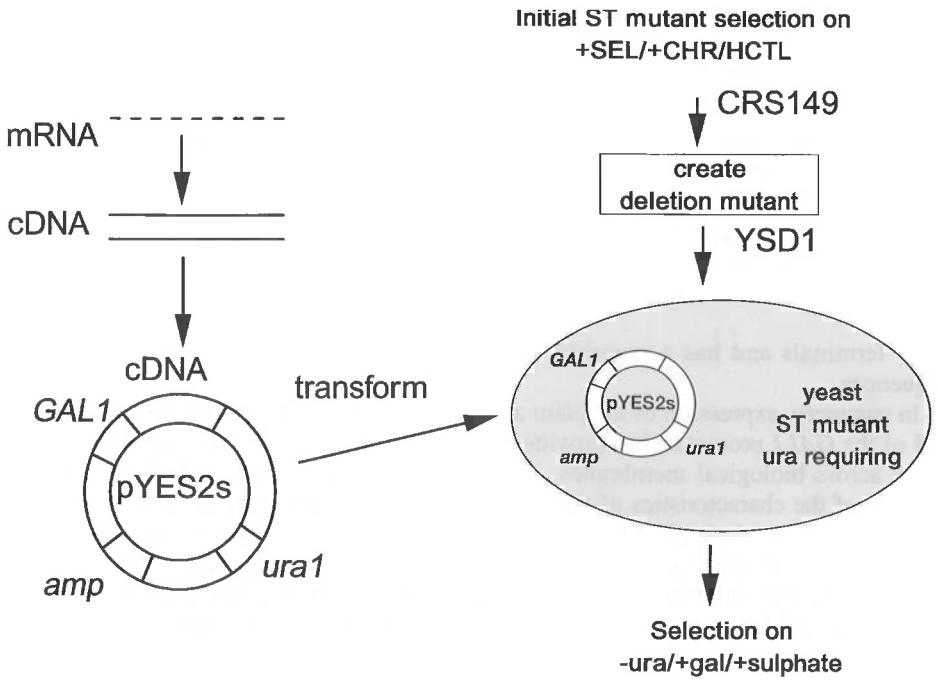


*Fig. 1.* Outline scheme for isolation of yeast sulphate-deficient mutants and their use to screen cDNA libraries for sulphate transporter genes. The pYES2 vector (Invitrogen), modified by substituting a *Sal* I site for the *Kpn* I site and renamed pYES2s (31), contains a galactose promoter (*GAL1*), an ampicillin-resistance gene and the *ura1* gene to complement the uracil requirement of the yeast strain. St:sulphate transporter, SEL:75μM selenate, CHR: 150μM chromate, HCTL: homocysteine thiolactone, CRS149: initial EMS generated mutant isolated, YSD1: yeast sulphate transporter deletion mutant.

and *hvst1*, respectively, and their respective protein products, SHST1, SHST2, SHST3 and HVST1. Tracer studies showed that all of these clones facilitated sulphate uptake into yeast strain YSD1 when expression was induced by growth on galactose. Furthermore, the characteristics of these plant sulphate transporters could be determined when expressed individually in the yeast YSD1 mutant. The affinities for sulphate were determined (Table 1) and all of the transporters showed simple, saturable Michaelis-Menton kinetics. The high affinity (SHST1, SHST2 and HVST1) transporters clearly represent versions of the high affinity transporter, the kinetics of which were first described by Leggett and Epstein (21) in 1956. However, SHST3 was unique amongst the cloned transporters in having a substantially lower affinity for sulphate, although probably not as low as the classically suggested low affinity mechanism II transporter (7) or the kind of apparently non-saturable uptake as can be observed in the yeast YSD1 mutant at mM sulphate concentrations (32). It is likely that SHST3 represents another sulphate transporter system, not usually detected in root influx experiments. Sulphate transport for all transporters was pH dependent in the pH range 5.6 to 7.6, being highest at lower pH, supportive of the idea that these are $H^+/SO_4^{2-}$ cotransporters (13).

Table 1 summarises the characteristics of the plant and yeast transporters. All appear to consist of single polypeptides, ranging in length from 644 to 859 amino acids with $M_r$s of 69800 to 96000. The predicted pIs indicate that the plant transporters are basic polypeptides. Sequence analysis indicates that SUL1 is longer at both the amino and car-

*Table 1.* Characterisitics of transporters cloned from plants and yeast

| Transporter | Organism | No. of amino acid residues | Predicted $M_r$ | Predicted pI | *K*m for sulphate (μM) |
|---|---|---|---|---|---|
| SHST1 | *Stylosanthes hamata* | 667 | 73100 | 9.28 | 10.0±0.6 |
| SHST2 | *Stylosanthes hamata* | 662 | 72700 | 9.10 | 11.2±0.5 |
| SHST3 | *Stylosanthes hamata* | 644 | 69800 | 9.10 | 99.2±4.8 |
| HVST1 | *Hordeum vulgare* | 660 | 72500 | 9.04 | 6.9±0.6 |
| SUL1 | *Saccharomyces cerevisiae* | 859 | 96000 | 6.35 | 7.5±0.6 |

boxy terminals and has a possible internal sequence which is absent from the plant sequences.

In summary, expression of the plant and yeast clones in yeast mutant YSD1 under control of the *GAL1* promoter, has provided direct evidence that these proteins transport sulphate across biological membranes. The yeast expression system will allow a detailed analysis of the characteristics of the plant transporters, in a system far more amenable to such studies than whole plant roots. Given that all of the genes facilitate sulphate transport across the yeast plasma membrane, it is likely that the natural role of all of these transporters is to transport sulphate across plasma membranes, and that they are not involved in intracellular sulphate transport, for example, across tonoplast or chloroplast membranes.

## A family of sulphate transporters

To date only the sequences of cDNAs encoding the three *Stylosanthes* sulphate transporters (31), an *Arabidopsis* cDNA (33) and the barley sulphate transporter have been reported (this review, Smith *et al.*, unpublished, Vidmar & Glass, this proceedings). Southern blot analysis suggests the presence of other homologous genes in *Stylosanthes* (31) and in barley (unpublished data, Smith *et al.*,) that are yet to be cloned. Comparison of the derived amino acid sequences (Fig. 2) suggests SHST1 and SHST2 to be almost identical (95% similar) but that SHST3 is a distinct sub-type (only 52% similar to shst1 and shst2). Based on restriction digest patterns, the origins of *shst*1 and *shst*2 have been suggested (31) to be from the two diploid progenitor species of the allotetraploidal *Stylosanthes hamata* cv. Verano. The derived amino acid sequence of the barley clone, HVST1, shows greatest similarity to SHST1 and SHST2 (69% similarity). Whilst there are substantial regions of identity between all three *Stylosanthes* sequences, the most notable difference is a substantial truncation of 25 amino acids from the amino terminus of SHST3. Throughout the sequence, there are clusters where the sequence homology is low, including much of the carboxy terminal hydrophilic region and some of the putative external facing extra-membrane loops.

The sequences of the plant sulphate transporters show strong similarity to several recently cloned sequences which form a novel transporter family. The plant transporters clearly show sequence similarity to the *N. crassa* and the yeast sequences. Sequence homologies between the *N. crassa cys-14* sulphate transporter and a number of genes not previously associated with sulphate transport were recognised recently (28). These included a human colon mucosa protein, whose expression is down-regulated in adenomas and adenocarcinomas, or *DRA* gene (29), and a nodule-specific protein (20). The *DRA* gene product has recently been shown to transport sulphate in *Xenopus* oocytes (30), however this has yet to be demonstrated for the nodule specific GMAKI70 protein.

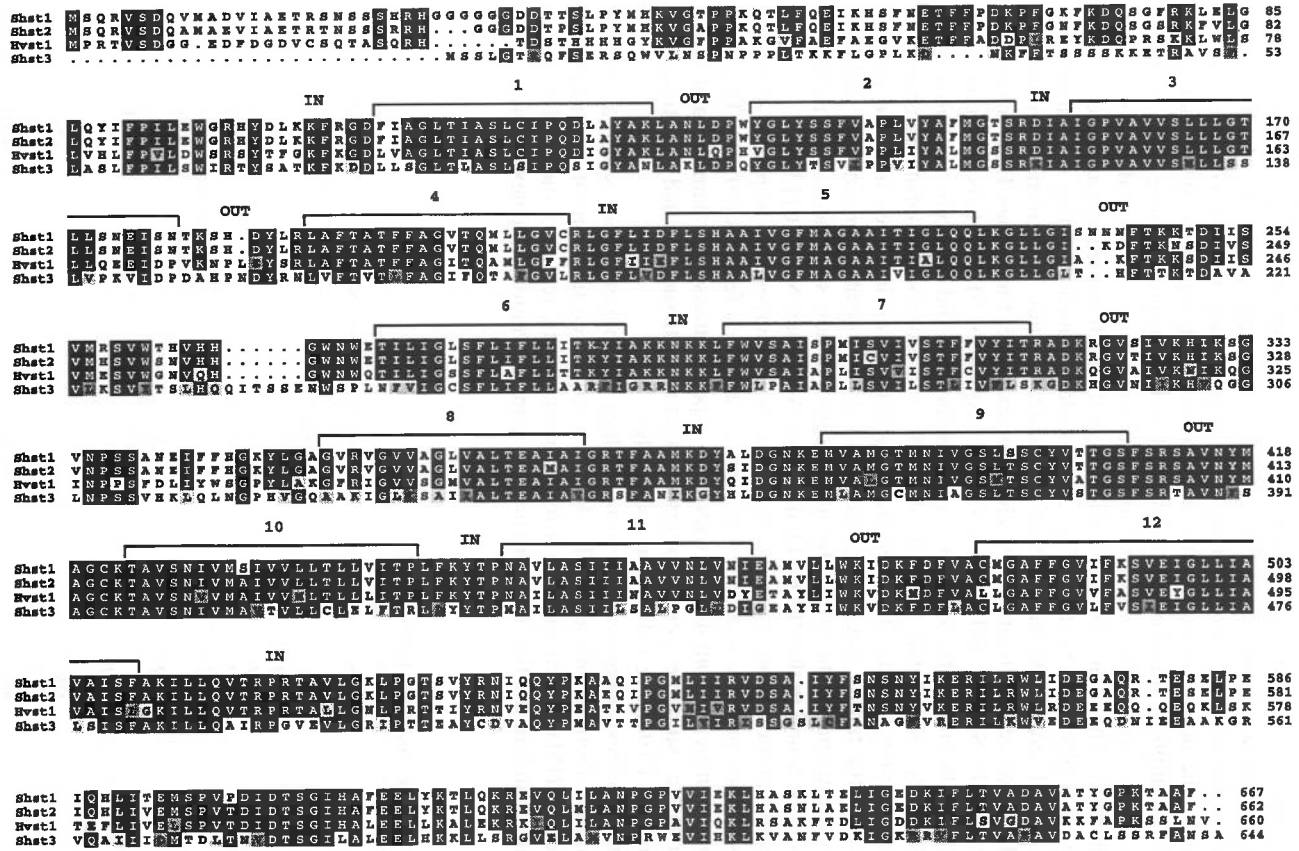


*Fig. 2.* Alignment of deduced amino acid sequences of SHST1, SHST2, SHST3 and HVST1 using PILEUP programme of the UWGCG package and displayed using PRETTY-BOX. Perfect identity is indicated by a black background and conserved substitutions by a gray background. The numbered horizontal bars indicate the position of the proposed membrane spanning domains (see text) and the proposed topology with respect to the cytoplasm of the extra-membrane loops is indicated: IN/OUT

More recent additions to this group are a human gene, *DTD*, a mutation in which results in diastrophic dysplasia (11), a rat liver $H^+$/sulphate transporter, *sat*-1 (1) and a mouse sulphate transporter (unpublished, see sequence database entry Accession No. D42049). The sequence databases also contain *Arabidopsis* EST fragments, a rice cDNA fragment and *Caenorhabditis elegans* fragments which show homology to this family. Table 2 lists all of the known members of the sulphate transporter family for which sequence information is known from gene cloning at the present date; this list will surely expand in the future. The degree of similarity of the amino acid sequences of the members of this family of sulphate transporters shows that these transporters have been conserved across eukaryotic organisms ranging from filamentous fungi, yeasts and plants to mammals and humans. The relationships of all of the full length sequences can be represented graphically as a phylogenetic tree (Fig. 3). It is clear from Fig. 3 that the mammalian, yeast and fungal, and plant members of the family fall into three distinct groups. The high similarity of certain members is clear, for example, the human DTD and mouse sequences, and the SHST1, SHST2 and HVST1 sequences. SHST3 falls into a quite distinct branch of the tree to the other *S. hamata* sequences and has some similarity to the GMAKI70 protein, which has yet to be shown to be a functional sulphate transporter. A considerable number of individual residues, or groups of residues are conserved amongst all members of the family, suggesting that they may be essential for function, and additionally providing diagnostic motifs for identification of new sequences. Such a diagnostic motif is to be found in the PROSITE database (Accession No. PS01130).

Membrane transport proteins from a wide variety of sources may be placed in distinct groups based upon primary sequence similarity or structural features (10, 25). This family shows no significant homologies with other reported transporter families and superfamilies or to $Na^+$/sulphate cotransporters from rat renal tissue (22) and ileal tissue (23) (Accession Nos. L19102 & U08031, respectively).

In addition to these putative eukaryotic sulphate transporters, there is also a potential sole prokaryotic member of the family. This is a sequenced fragment of an unidentified *E.coli* gene (YCHM), which has similarity to the diagnostic region of the sulphate transporter family found in the first three membrane spanning regions (see next section).

*Table 2.* The sulphate transporter family

| Organism | Designation of gene | Accession number | Notes | Reference |
|---|---|---|---|---|
| *Stylosanthes hamata* | *shst1* | X82255 | – | (31) |
| *Stylosanthes hamata* | *shst2* | X82256 | – | (31) |
| *Stylosanthes hamata* | *shst3* | X82454 | – | (31) |
| *Hordeum vulgare* | *hvst1* | X96431 | – | This review |
| Soybean | *GMAKI70* | D13505 | nodule specific | (20) |
| *Arabidopsis* | | T21459,T44718 | fragments | EST database |
| *Arabidopsis* | *AST56* | D85416 | | (33) |
| Rice | – | D25000 | fragment | EST database |
| *Saccharomyces cerevisiae* | *SUL1* | X82013 | – | (32) |
| *Neurospora crassa* | *cys-14* | J05321 | – | (18) |
| Human | *DRA* | L02785 | – | (29) |
| Human | *DTD* | U14528 | – | (11) |
| Mouse | *mmstob* | D42049 | – | EMBL database |
| Rat | *sat-1* | L23413 | liver | (1) |
| *Caenorhabditis elegans* | *W04G3.6,F14D12.5* | U41021, Z68014 | – | EMBL database |
| *Escherichia coli* | *YCHM* | | fragment | P40877 |

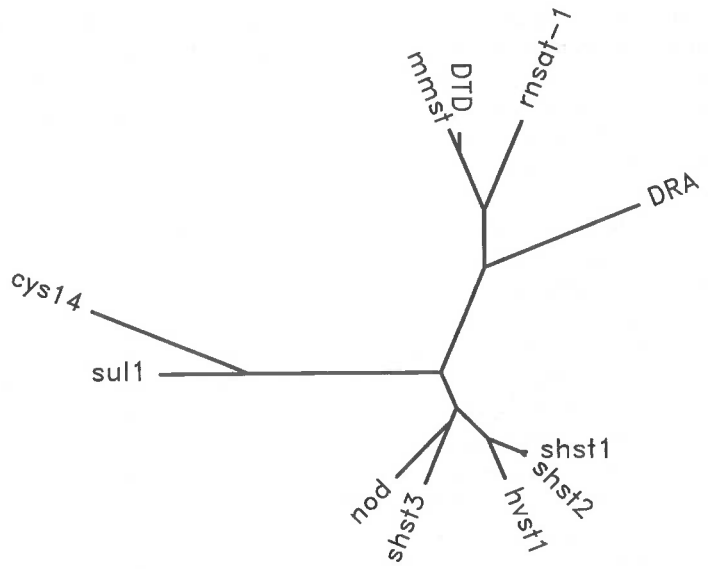


*Fig. 3.* Phylogenetic relationships between members of a family of eukaryotic sulphate transporters. The 11 full-length deduced amino acid sequences listed in Table 2 were aligned using PILEUP and phylogenetic distances calculated using PROTDIST (in PHYLIP) and plotted as a phylogenetic tree.

## Topology model of the sulphate transporters

All of the members of the family have been analysed for the occurrence of membrane spanning domains (MSDs) by assessment of hydropathy profiles, analysis of individual sequences using MEMSAT (16), TMAP (24) and PHDhtm (27). Simple hydropathy analysis clearly shows the overall hydrophobic nature of the sequence, with the exception of the N- and C-terminal regions. Within the central regions of each of the sequences 10 to 14 areas of high hydrophobicity can be identified, many of which are common to all sequences of the family.

Many cation/solute symporters examined to date have been suggested to contain 12 MSDs (see 10). Predictions of 12 MSDs for the sulphate transporter family were made using the MEMSAT program (16) and a consensus of 11 is readily achieved. In such a closely conserved family, it is likely that the major structural features, such as positioning of MSDs, would be conserved, and although predictive algorithms fail to find a consensus for the 12th MSD, it is highly probable that, if there is a 12th MSD, it would be conserved in all members of the family.

The most sophisticated analysis (PHDhtm) utilises an alignment of members of the family and applies a range of rules and observations to predict the most likely consensus topology model (27). This algorthim produces a topology which agrees well with that produced by other methods but fails to predict MSD 9. This is a very hydrophobic region, contains no charged residues and is consistently predicted by MEMSAT. A further problem with an 11 MSD model is that the carboxyl terminal is predicted to be extracellular, which would be an unusual feature. For these reasons, the model presented adheres to the generally accepted principle of 12 MSDs for this type of solute co-transporter.

The positions of the tentative hydrophobic MSDs indicated agree closely with those proposed by Hästbacka *et al.* (11) for the rat liver sulphate transporter (*sat-1*) and the

human *DTD* and *DRA* gene products. Hästbacka *et al.* (11) also propose a carboxyl-terminal membrane-associated region, a relatively hydrophobic region also present in the plant sequences. The potential glycosylation site present in all of the mammalian sequences between helices 3 and 4 is not present in any of the plant or fungal sequences and the alignments introduce a gap in this region.

The topology diagram (Fig. 4) indicates the positions of the helices relative to the SHST1 sequence. Although the majority of charged residues are in the internal loops, as would be expected from the "positive inside" rule (34), an unusually high number of positive residues are found in the predicted extracellular loops, particularly in the loop between MSD 7 and MSD 8. These observations are similar to those made on the *N. crassa* sulphate transporter (5), but the greater number of sequences now available has given rise to a predicted topology modified from that previously described (5, 32). The very high density of arginines in the coding sequence may be due to a mistaken assignment of exons in the central region of the *N. crassa* sulphate transporter gene, as the *Neurospora* sequence fails to show any homology with any of the other sulphate transporter sequences in this region. Clarkson *et al.* (4) demonstrated the sensitivity of the barley sulphate transport system to a membrane impermeant arginine-specific reagent, hydroxyphenylglyoxal, indicating the presence of essential arginine residue(s) accessible from the extracellular surface of the plasma membrane. The predicted model includes a number of extracellular arginines in the *S. hamata* sequences, only one of which, between MSDs 9 and 10, is conserved in all sequences. Six charged residues appear within the predicted MSDs; it is tempting to speculate that they may have a role in ion translocation.

## Expression of sulphate transporters in relation to sulphur nutrition

Northern blot analysis has clearly demonstrated that *shst1*, *shst2* and *hvst1* are all expressed exclusively in root tissues (Fig. 5). Furthermore, in mRNA preparations extracted from tissues subjected to several days without an external sulphate supply, the pool-sizes are considerably enhanced (31). In the same plant materials mRNA hybridizing to a *shst3* probe can be detected in preparations from both root and shoot (Fig. 5). It may also be the case that the mRNA pool-size of *shst3* in the shoots decreases during S-starvation, and is slightly enhanced in roots. In all cases the abundance of *shst3*-hybridizing message is considerably lower than the *shst1/2* hybridising message (31), although it is not known to what extent this is reflected in the relative abundances of the various transporter proteins.

In derepression/re-repression experiments, plants are first subject to several days S-starvation, during which the capacity for sulphate uptake increases many-fold (typically from 100 to 1500 nmol $g^{-1}$ $h^{-1}$ over 4 days) (for example 6, 12). During this time, the abundance of the mRNA pool for *shst1/2* in *Stylosanthes* and *hvst1* in barley continues to increase (Fig. 6). During the subsequent re-repression phase, when sulphate is added back to the culture medium, the uptake capacity falls rapidly with a half-time of a few hours, the mRNA pool-size also falls between 2 and 8 hours to a very low level, but then recovers back to the typical "repressed" abundance (Fig. 6). In other experiments, a substantially decreased pool-size has been apparent in barley after only one hour (Hawkesford, unpublished observations) and which supports the half-life determined for the transporter in tobacco cells (26). For this rapid fall to be due to regulation of gene expression alone would require there to be a very rapid turnover of protein in the membrane and for there to be only a small pool in transit to the plasma membrane. The mRNA pool for the transporter would also have to be rapidly degraded unless it is usually turned over rapidly.

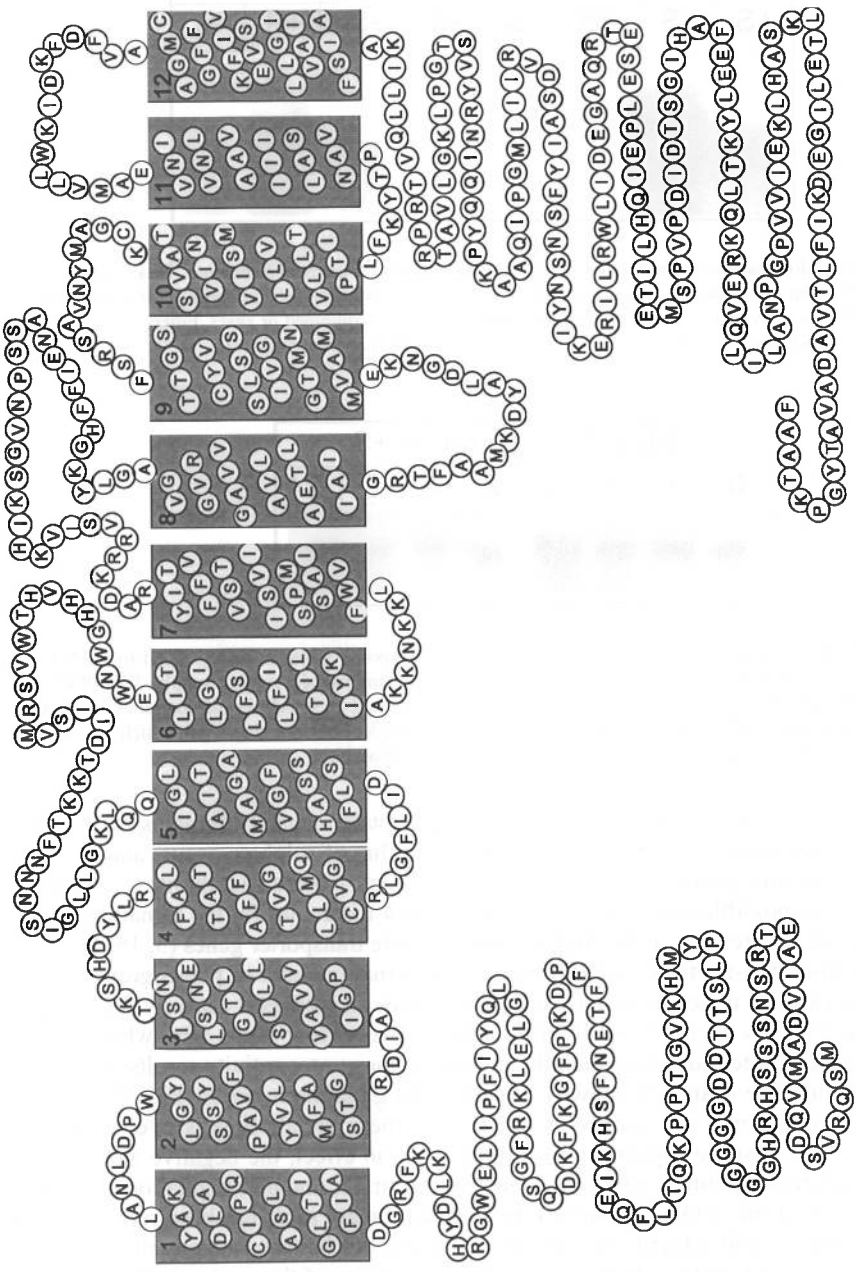

*Fig. 4.* Proposed topology of SHST1 sulphate transporter. Twelve membrane-spanning domains are predicted and the amino and carboxyl terminal regions are proposed to be cytoplasmically-located. No data is available on the conformation of the extra-membranous loops and any indicated structure is purely diagrammatic. MSDs were computed by a combination of hydropathy profile analysis, MEMSAT (16), PHDhtm (27) and TMAP (24).

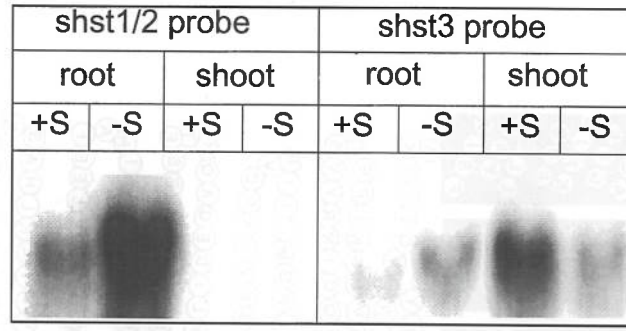


*Fig. 5.* Northern blot analysis of expression of the sulphate transporter genes in *S. hamata*. Total root RNA and total leaf RNA from *S. hamata* plants grown under normal sulphur nutrition (+S) or deprived of sulphate for 72 hours (-S). RNA was hybridized with probe from either *shst1/shst2* combined or *shst3*. Filters were washed at high stringency and exposed to X-ray film for 24 hours (*shst1/shst2*) or 6 days (*shst3*).

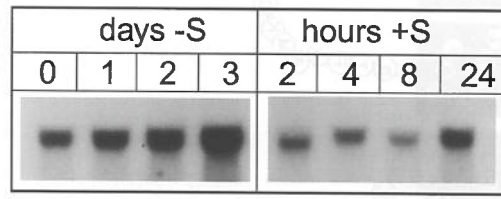


*Fig. 6.* Northern blot analysis of expression of the sulphate transporter genes in barley. Total root RNA was extracted from plants prior to, and 1,2, and 3 days following, the removal of a sulphur supply. Total RNA was extracted from roots 2,4,8 and 24 hours after sulphate was re-supplied (1.5 mM). Equal quantities of RNA (as judged by visualisation rRNA stained with Methylene Blue) were loaded on a single gel, which was blotted, hybridised and exposed together, so that all tracks are directly comparable.

During these first few hours of repression, when uptake capacity remains high in the presence of high external sulphate, a considerable influx of sulphate occurs and root sulphate, cysteine and glutathione pools rise. All three of these metabolites, or related metabolites, are possible candidates to act as repressor molecules for the signalling which inhibits specific expression of the high affinity sulphate transporter genes (3, 15, 17).

The addition of O-acetyl serine (OAS) to the external medium of plants growing with an adequate external concentration of sulphate induces an increased capacity for sulphate uptake (Fig. 7). This occurs typically after a lag of two to four hours after which a 3- to 4-fold increase is noted, during which the increased transporter activity results in a rise in the internal sulphate (data not shown), cysteine and glutathione pools (Fig. 7). Clearly, under these conditions, any repressor function of these metabolites is over-ridden by OAS acting as a positive regulatory metabolite, and, in effect, the negative and positive regulators act in competition with one another. In a similar manner, regulation of expression of activity of the ATP sulphurylase in cultured tomato roots has been reported (14). Such a situation would directly parallel the regulatory mechanisms suggested to occur in *E. coli* (17), in which the CysB protein modulated activity of the cys promoter is dependant upon its interaction with either OAS or sulphide. These control mechanisms may be related to those occurring in yeast and fungi in which the regulation is likely to be linked to methionine or some related metabolite, and is effected by the products of control genes such as *met4* and *met28* (see for example Surdin-Kerjan, this volume) and *cys-3* (9, 19).

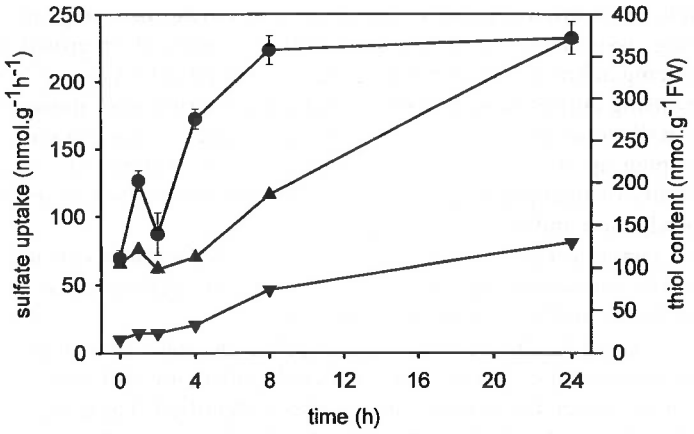


*Fig. 7.* Influence of O-acetyl serine (OAS) on sulphate uptake and thiol content of barley roots. OAS (0.2 mM) was added to10-d-old plants, maintained on 0.15 mM sulphate, and uptake capacity during a 10 minute period (•), cysteine (▼) and glutathione (▲) contents were determined prior to, and 1, 2, 4, 8 and 24 hours after OAS addition. Thiols were determined by HPLC (35). All data are the means of at least three replicates (6 replicates for transporter activities) with standard error bars.

Alternatively, in addition to regulation of expression, there may be direct allosteric regulation of the transporter protein by sulphate or some metabolite which could provide a fine level of control continuously modulating transporter activity. This may be reflected in the rapid decrease in transporter activity observed after sulphate re-addition. However, the OAS experiment would indicate that OAS activation can overcome this inhibition; any complete explanation would need to take this into account.

## Future directions

The availability of cDNA clones for plant sulphate transporters has already begun to answer many long standing questions in plant sulphate transport research and will help in many more general studies of plant sulphur metabolism and plant nutrition. Much remains to be done on the localisation of the transporters, both the root specific transporters in relation to the root tip, root epidermal and cortical cells, and the role of root hairs. The elucidation of the exact site of expression and the role(s) of the globally-expressed isoform, of which *shst3* is currently the only example, are not yet clear. *In situ* hybridisation, immuno-localisation and reporter gene studies should clarify these questions. The cloned genes, and fragments derived from them, have already been subcloned into over-expression vectors (see Schneider and Hawkesford, this volume) and antibodies are being produced to these fragments. Choice of fragments with low homology should enable discrimination between sub-types of these transporters.

Site-directed mutagenesis will probe the structure-function relationships of the transporter and such studies have commenced to evaluate the role of arginine residues in the transport processes. Utilisation of the YSD1 mutant facilitates the analysis of these mutants providing a very valuable functional expression system for the analysis of sulphate transporter kinetics.

Transgenic plants, in which attempts are being made to manipulate the expression of plant sulphate transporter clones, are being generated. These plants contain constructs

which uncouple the normal regulatory processes that synchronise sulphate uptake, storage, partitioning, assimilation, and the use of sulphur compounds in growth and development. This is being achieved by overexpressing introduced cDNAs using a range of promoters and by using antisense technology to reduce transcription of transporter mRNAs. Such studies will generate a wealth of new knowledge on the regulatory processes involved in sulphur uptake and utilisation by plants. It will also provide valuable insights into the feasibility of manipulating sulphate uptake and metabolism in order to improve agricultural production and the quality of plant products.

Isolation of genomic clones and analysis of promoter regions will hold the key to understanding the control of expression by nutrition and environmental factors and the interactions of the N- and S- assimilatory pathways.

In spite of the considerable recent advances in the cloning of the plant plasma membrane sulphate transporters, it may be speculated that there are still many other transport steps for which the molecular species have not been identified. These may include transporters related to those already described but specifically responsible, as an example, for loading and unloading sulphate into and out of the xylem or for the transport of sulphate into specific tissues, such as developing grains. There may also be quite unrelated sulphate transporters remaining to be "discovered", notably the transporter responsible for sulphate influx into the chloroplast, the major site for sulphate reduction, and the transporters responsible for both influx and efflux from the vacuole, the major storage site for sulphate. These are all major goals for future research.

## Acknowledgements

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# REGULATION OF GENE EXPRESSION IN YEAST SULPHUR METABOLISM

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## Abstract

The metabolism of sulphur amino acids in *Saccharomyces cerevisiae* is a paradigm for the study of the regulation of transcription in eukaryotes. All the genes encoding the enzymes catalysing the sulphate assimilation pathway are transcribed coordinately and submitted to a specific negative regulation: their transcription is turned off in response to an increase in the intracellular concentration of the final product of the pathway, S-adenosylmethionine (AdoMet),

The transcriptional regulation of this pathway is known to require four different factors: the Centromere Binding Factor 1 (Cbf1), a basic helix-loop-helix-leucine-zipper protein, two basic-leucine-zipper proteins, Met4 and Met28, and one WD40 factor, Met30. In contrast to Cbf1 and Met28 wich are devoid of transcription activation function, Met4 behaves as a strong transcription activator responsive to intracellular AdoMet. In addition to its bZIP domain, Met4 is comprised of three functional regions: one acidic activation domain and two regulatory domains involved in the AdoMet inhibition of the activation domain. The AdoMet mediated inhibition of Met4 requires the Met30 factor which was shown to function as a transcriptional inhibitor and to directly interact with Met4. We have recently demonstrated that the transcription activation of the sulphur metabolism actually requires the assembly of a highly organized heteromeric complex containing Cbf1, Met4 and Met28. Two hybrid studies have allowed us to investigate the binary protein-protein interactions involved in the Cbf1 / Met4 / Met28 complex assembly: they show that the leucine zippers of Met4 and Met28 along with the bHLH domain of Cbf1 provide the protein surfaces responsible for the assembly.

## Introduction

Transcriptional regulation of gene expression can be achieved through either activation or repression mechanisms. The biosynthesis of sulphur amino acids in the yeast *Saccharomyces cerevisiae* constitutes one metabolic pathway negatively regulated at the level of transcription. The pathway including sulphate transport, its reduction and the assimilation of the sulphur atom (the sulphate assimilation pathway) involves the products of eight unlinked genes the expression of which is regulated by S-adenosylmethionine (AdoMet) (figure 1): the transcription of the sulphur genes is turned off in response to an increase in the intracellular level of AdoMet (17, 20).

The deletion analysis of the promoter region of one sulphur gene, *MET25*, has shown that it comprises two motifs involved in the regulation of transcription of the gene: one is the palindromic sequence TCACGTG which can be found once or twice in the promoter region of each sulphur gene and which is necessary for transcription activation and the second one is the sequence GAAAATGTGG, necessary for the complete repression of transcription (17, 6).

In contrast to this apparent simplicity, at least four transcriptional factors are involved in this regulation. They comprise two members of the basic-leucine-zipper (b-ZIP) family, Met4p and Met28p, one member of the basic-helix-loop-helix-leucine-zipper (bHLH-LZ) family and one member of the family of proteins bearing WD40 motifs.

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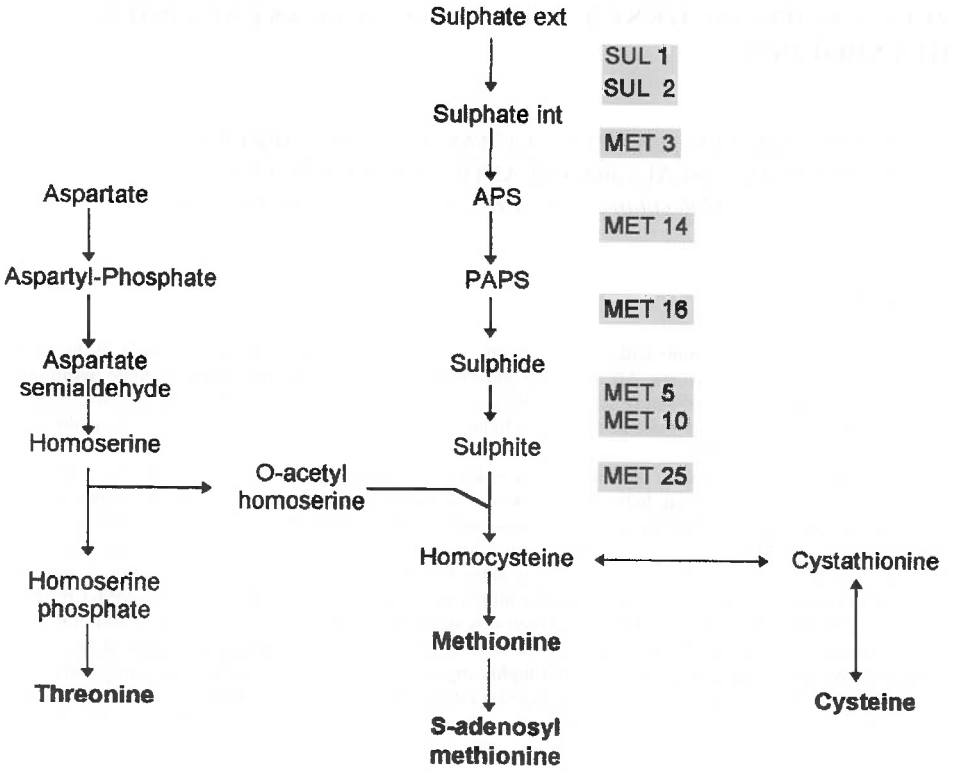


*Fig. 1.* Biosynthesis of sulphur amino acids in *Saccharomyces cerevisiae.* (APS: adenylyl sulphate, PAPS: 3-phospho-adenylyl sulphate).

Here we will show that the transcription of the sulphur genes results from the formation of a high molecular weight heteromeric complex involving Met4p, Met28p and Cbf1p. In addition, a genetic analysis of sulphate transport has produced evidence for two genes encoding two sulphate transporters and one gene encoding a new regulatory factor.

## Results

*Regulation of the activation functions of Met4p.*

The Met4 protein comprises 666 amino acid residues and bears at its C-terminal a basic-leucine-zipper (b-ZIP) motif. To test whether Met4p could activate transcription, we used a LexA-Met4 fusion protein containing the DNA binding domain of the bacterial repressor LexA. The ability of the LexA-Met4 protein to activate transcription was tested by using a target plasmid bearing a *GAL1-LacZ* reporter gene carrying LexA binding sites upstream the transcription start site (3). The LexA-Met4 fusion protein is capable of efficiently activating the transcription of the reporter gene, showing that Met4p bears transcriptional activation functions (18). In addition, this activation function is one target of the negative regulation as it is specifically inhibited by an increase of the intracellular concentration of AdoMet. To identify the regions of Met4p capable of activating tran-

scription as well as the regions conferring AdoMet responsiveness, we constructed several series of internal deletions in Met4p. Each derivative was expressed as a fusion with the DNA binding domain of LexA and introduced on a plasmid in a strain in which the *MET4* gene has been deleted (*met4::TRP1*). This strain bore also a LexAop-LacZ fusion gene and the level of β-galactosidase activity was used as a measure of the transcriptional capability of each modified Met4 protein. It is noteworthy that in this case the DNA binding of the different Met4 derivatives is achieved through the LexA moiety, but in all cases, the b-ZIP domain of Met4p was conserved so as to preserve the possibility of complementing the methionine auxotrophy of the *met4::TRP1* strain.

The results obtained by the analysis of about sixty Met4 derivatives show that, in addition to the b-ZIP domain, Met4p bears three domains of functional importance, responsible for the transcriptional activation function and its regulation (figure 2). Met4p comprises a unique activation domain located in its N-terminal region between residues 95 and 144. This domain has a high content of acid residues and of asparagines and functions in a constitutive manner. The inhibition of the transcriptional activation function requires a domain located between residues 189 and 235 that we called the inhibitory domain. The function of the inhibitory domain is not specific to the activation domain of Met4p. Indeed, experiments using LexA-Gal4-Met4 fusion proteins show that it can inhibit the transcriptional activation function of the Gal4 activation domain in response to an increase of the intracellular concentration of AdoMet (9). This shows that an unrelated heterologous activation domain can be negatively controlled by AdoMet if the inhibitory region of Met4p is present.

Our analysis showed also the existence of another domain within the Met4 residues 312 to 375, that we called the auxiliary domain. The deletion of this region reduces the transcriptional activation function of Met4p threefold under non repressive conditions. The analysis of the results obtained with the fusion proteins deleted from this auxiliary region as well as experiments with Gal4-Met4 fusion proteins show that this auxiliary domain is devoid of intrinsic transcriptional capacities. The role of this auxiliary domain was elucidated by experimants using Gal4-Met4 fusion proteins which show that in the absence of this region the inhibitory region prevents to a large extent the activity of the activation domain, even when the intracellular concentration of AdoMet is low (9). Addition of the auxiliary domain relieves this negative effect, thus appearing as an antagonist of the inhibitory region under non-repressive growth conditions. Taken together, these results allowed us to propose a model for the regulation of the functions of Met4p and illustrated in figure 3. When the intracellular concentration of AdoMet is high, the inhibitory region would govern an interaction with an "inhibitory protein" which would prevent the activation domain from contacting the the transcription machinery. When the AdoMet level is low, the reversal of the of the inhibition of the activation domain would be facilitated by the auxiliary domain.

**The transcriptional inhibitor Met30p is a WD40 protein.**

The model that we proposed for the regulation of the activation function of Met4p postulated the existence of an "inhibitory protein". Using a specific screen involving gene *XylE* from *Pseudomonas putida* encoding catechol oxidase, an enzyme which is not synthesized by yeast, we have isolated mutants in a gene that we called *MET30*. These mutants are characterized by a lowered transcriptional inhibition of the sulphur genes (table 1).

Gene *MET30* was cloned and the analysis of its sequence showed that it bears five WD40 motifs. Proteins bearing WD40 motifs are eukaryotic proteins and form a grow-

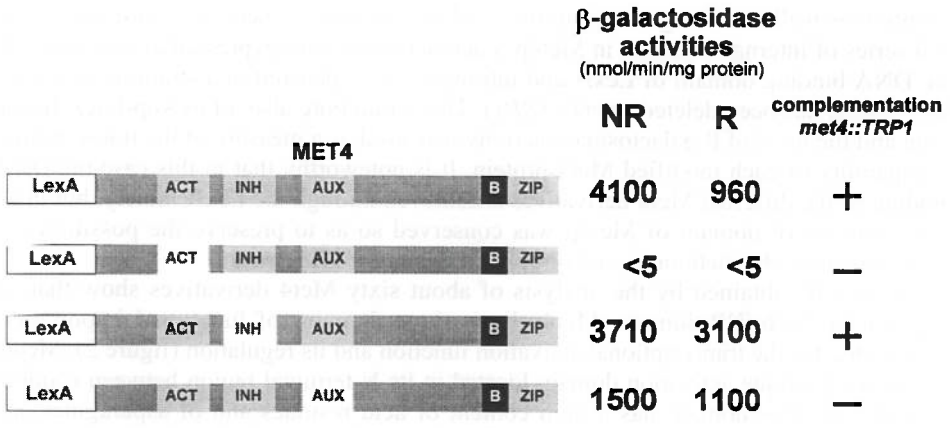


*Fig.2.* The functional domains of Met4p, the transcription activator of the yeast sulphur genes. Schematic representation of the fusions between the DNA binding domain of LexA and various derivatives of Met4p are shown. Plasmids expressing these fusion proteins were introduced into a *met4* disrupted strain also bearing a lexAop-lacZ reporter gene integrated into a chromosome. The β-galactosidase activities were measured in cells grown in non-repressive (NR, 0.05 mM L-methionine) or repressive (R, 1 mM L-methionine) growth conditions. The reported values are the averages of at least three assays performed with different transformants. The ability of each plasmid to complement the methionine auxotrophy of a *met4::TRP1* mutant is shown.

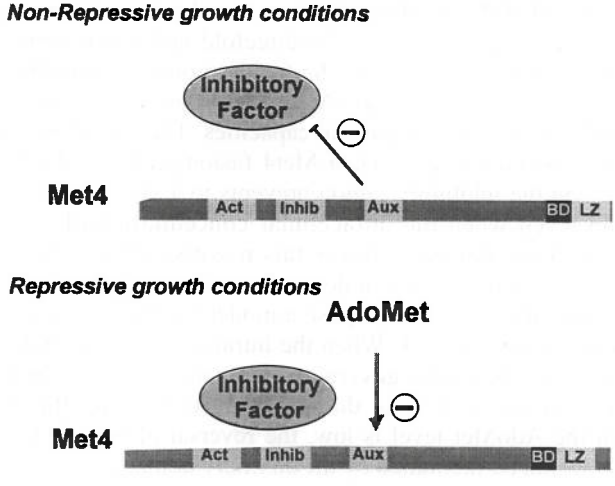


*Fig.3.* Proposed model for the intra-molecular regulation of Met4p (for details, see text).

*Table 1.* The *met30-1* mutation affects the sulphate assimilation pathway. Specific activities of two enzymes involved in the sulphate assimilation pathway were assayed in a wild type (*MET30*) and a *met30-1* spore from the same tetrad grown in either non-repressive (no methionine) or repressive (1 mM L-methionine) growth conditions. All the enzymatic activities are expressed in nmol of substrate transformed per min and per mg protein. Data are the averages of two independent experiments (deviation was less than 10 %).

| Strain | Relevant genotype | Growth condition | Homocysteine synthase activity | ATP sulphurylase activity |
|---|---|---|---|---|
| CM100-1A | *met30-1* | Non-repressive | 410 | 168 |
| | | Repressive | 208 | 40 |
| CM100-1B | *MET30* | Non-repressive | 254 | 150 |
| | | Repressive | 62 | < 5 |

ing family, the members of which seem to have a regulatory function.These motifs are believed to be implicated in protein-protein interactions (13, 14). Met30p displays strong similarity to Scon2p, the negative regulatory factor involved in the regulation of the sulphur genes in *Neurospora crassa*. In addition to the WD40 repeats, Met30p and Scon2p exhibit in their N-terminal region a motif characterized by several strongly conserved charged residues and an invariant Leu-Pro dipeptide (7). We have shown that the AdoMet inhibition of the transcriptional activation function of Met4p is dependant on the presence of Met30p (figure 4). Then, using the "two hybrid" system, we have shown that Met4p and Met30 interact *in vivo* and that these interactions were considerably lowered when the inhibitory region of Met4p was deleted (figure 5).

Taken together, all our results show that Met30 exhibits all the properties of the "inhibitory factor" postulated by the model of inhibition of the transcription activation functions of Met4p (figure 3). It is however highly probable that Met30 interacts with other transcriptional activators as the inactivation of gene *MET30* results in non-viable strains. Indeed, we have shown that mutations in gene *MET30* modify the regulation of the synthesis of glucose-6-phosphate dehydrogenase (Thomas *et al*, unpublished results) and that of AdoMet synthetases (19). This result is compatible with what is known on WD40 motifs as it has been shown that one motif is enough to interact specifically with one other protein (5).

**The general factor Cbf1 (Centromere binding factor 1) is implicated in the transcriptional activation.**

The TCACGTG motif found in the promoter region of all sulphur genes, which is required for the activation of the transcription of these genes, has been shown to participate in another major cellular process: the segregation of chromosomes. Indeed, this motif has the same consensus sequence as CDE1, a conserved element of the *S. cerevisiae* centromeres. Three different laboratories have isolated the gene encoding the protein which binds to CDE1 (1, 2, 12). This protein, Cbf1 (for Centromere binding factor 1), belongs to the family of proteins that bind to DNA through a b-HLH-LZ motif. However, Cbf1 has no transcriptional activation capacities (18). The inactivation if gene *CBF1* has two phenotypic consequences: it results in the increase of the chromosome instability and in an auxotrophy for methionine. By analysing the kinetics of transcription of the sulphur genes we have identified the genes the transcription of which is partially or strictly dependant on the integrity of gene *CBF1* (10). A model in which Cbf1p functions by triggering chromatin opening, thereby facilitating the binding of specific transcription activa-

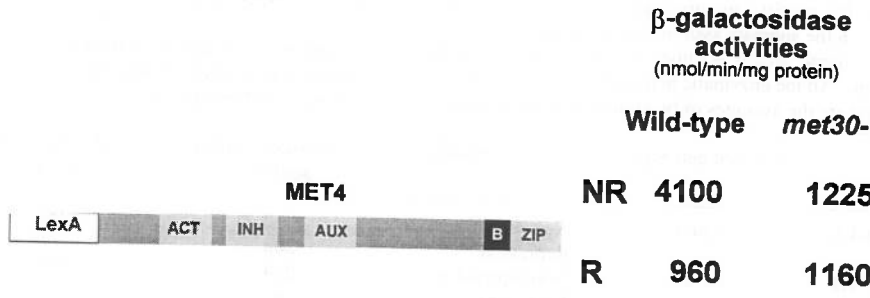


*Fig.4.* The inhibition of the transcription activation function of Met4p depends on Met30p. The plasmid encoding the lexA-Met4 fusion protein was transformed into the *met30-1* mutant and its parental wild-type strain, both bearing the lexAop-lacZ reporter gene. The β-galactosidase activities were measured in cells grown in non-repressive (NR, 0.05 mM L-methionine) or repressive (R, 1 mM L-methionine) growth conditions. The reported values are the averages of at least three assays performed with different transformants.

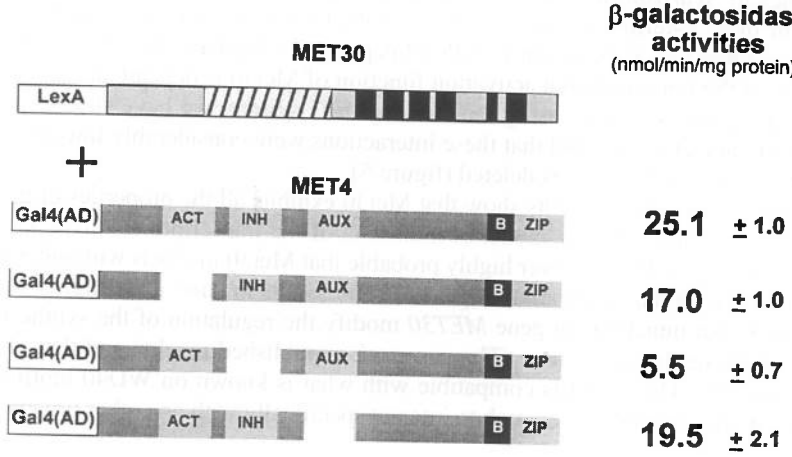


*Fig.5.* Interactions between Met30p and Met4p measured in the "two hybrid" system*. Strain C170 (*met4::TRP1*, *ura3::lexAop-lacZ::URA3*) was co-transformed with a plasmid expressing the lexA-Met30 fusion protein and different plasmids expressing various Gal4(AD)-Met4 fusion proteins (Gal4-AD corresponds to the activation domain of the yeast transcription activator Gal4). The cells were grown in non-repressive growth conditions (0.05 mM L-methionine). Data are the average of four assays performed with independent transformants. * Schematic representations of the fusion proteins. On Met30, the black boxes indicate the WD40 motifs and the hatched box represents the "MAD" domain (see Kumar and Paietta, 6).

tors had been postulated by Mellor *et al.* (12). However, the recent analysis of the nucleosome organisation of the *MET16* upstream region in a wild type and in a *cbf1* null mutant revealed only minor differences around the CDE1 sites (4, 15). The role of Cbf1p in the activation of the sulphur genes expression thus remained to be determined.

**Another b-ZIP protein, Met28p is involved in transcriptional activation.**

Using a specific genetic screen based on resistance to selenate, a toxic analogue of sulphate, we have recently identified a new gene, *MET28*, that encodes a protein also involved in the regulation of the transcription of the sulphur genes. Cloning and sequence analysis of this gene has shown that it encodes a small protein (166 residues) which belongs also to the b-ZIP family. To examine the role of Met28p, we constructed a fusion between the DNA binding domain of LexA and Met28p, and tested the capability of the fusion protein to activate the transcription of gene *LacZ* placed downstream of *LexA* operators. Results (figure 6) show that the LexA-Met28 fusion activates weakly the transcription of gene *LacZ* when it is expressed in a wild type strain. This activation depends on the presence of Met4p as shown by the lack of activation by the LexA-Met28 protein when expressed in a *met4* disrupted strain. This can be interpreted as an indication that the DNA bound LexA-Met28 fusion protein recruits the Met4 protein which provides the transcriptional activation function. The Met28 protein thus appears to possess no transcriptional activation function. This figure illustrates also that Cbf1p has no transcription activation capabilities.

**Met28p binds to the promoter region of gene *MET16* as part of a high molecular weight complex.**

This result along with the analysis of the amino acid sequence of Met28p predicted its ability to bind to DNA. To examine this possibility, we performed mobility shift assays with extracts from strains bearing mutations in *CBF1*, *MET4* or *MET28* genes and a probe specific to the *MET16* gene containing the Cbf1p binding site that had been shown to be required for transcription activation (15). Gel retardation assays performed with an extract from a wild type strain display one main complex corresponding to the binding of Cbf1, as it disappears when an extract fron a *cbf1::TRP1* mutant is used. A second complex of lower mobility is also present in extracts from a wild type strain but is not seen when extracts from *cbf1*, *met4* or *met28* deleted strains are used. To determine if Met28p is present in this complex, we used an extract from a *met28::URA3* mutant transformed by a plasmid expressing a LexA-Met28 fusion. In this case, the low mobility complex is seen. Addition of anti-LexA antibodies to this extract results in a complex of lower mobility (supershift). These results show that a high molecular weight complex binds to the probe and that Met28p is a component of the complex (8).

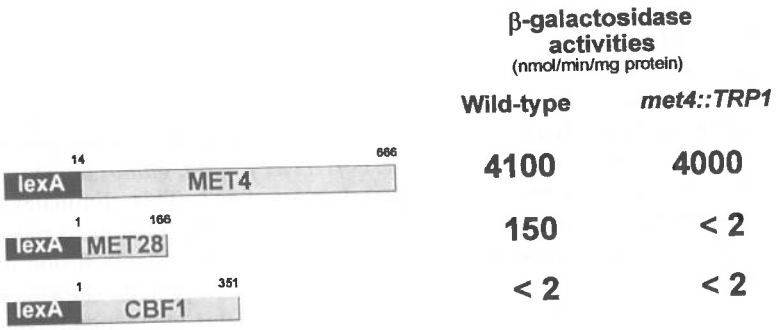


*Fig.6.* Only Met4p behaves as a transcriptional activator. For experimental details, see legend of Fig.2.

**A Cbf1/Met4/Met28 heteromeric complex mediates the transcription activation of the sulphur genes.**

The experiments described above revealed that Cbf1 and Met4p are required for the formation of the complex containing Met28p. However, they did not formally show the presence of Cbf1p and Met4p in the complex. To examine this possibility, mobility shift assays were performed with extracts of *cbf1::TRP1* and *met4::TRP1* mutants transformed with plasmids expressing respectively a LexA-Cbf1 and a LexA-Met4 fusion protein. In both cases, the low mobility complex is seen when the extracts of the transformed strains are used. Once more, the addition of anti-LexA antibodies to the two extracts results in a supershift of this complex. As expected, when the antibodies are added to the *cbf1* disrupted mutant expressing the LexA-Cbf1 fusion protein, a supershift of the main complex corresponding to the binding of the LexA-Cbf1 fusion protein alone is also seen. All these results indicate that both Cbf1p and Met4p are components of the high molecular weight complex which appears to be composed of at least three different proteins. It must be noted that these experiments reveal no complex that would correspond to the binding of Met4p or Met28p alone.

To get a better insight into the function of Met28p, it was expressed in *E coli* as a glutathione-S-transferase fusion protein (GST-Met28) and purified. The DNA binding characteristics of the fusion protein were analysed also by mobility shift assays. The GST-Met28 fusion protein binds to the *MET16* probe. However, the addition of non-specific competitor DNA eliminates the DNA binding activity of the GST-Met28 fusion protein, suggesting that this protein binds to the *MET16* probe with a low affinity. The GST-Met28 fusion protein was used in combination with extracts from different mutants. The combination of the GST-Met28 fusion protein with an extract from a *met28::URA3* mutant stimulates the formation of a low mobility complex even in the presence of the competitor DNA. This complex is not seen when the GST-Met28 fusion protein is used in combination with extracts from either *met28::URA3, cbf1::TRP1* or the *met28::URA3, met4::TRP1* double mutants. These results show that the presence of Cbf1p and Met4p stimulates the DNA binding activity of the GST-Met28 fusion protein through the formation of a high molecular weight complex (8).

**Protein-protein associations in the Cbf1/Met4/Met28 complex**

To investigate the interactions governing the assembly of the complex, we used the two hybrid system, that allows one to study *in vivo* protein-protein interactions by assaying the ability of two protein fusions to reconstitute a transcription activator through protein-protein interactions. Cbf1, Met4 and Met28 were thus expressed as fusion proteins either with the DNA binding domain of LexA or the activation domain of Gal4, the yeast transcriptional activator of the *GAL* system. All the fusion proteins retained the capacity to functionally complement the methionine auxotrophy associated with their respective disruption mutation and can thus be considered to be functional. The fusion proteins were expressed in strain CC800-16C (*met4::TRP1, cbf1::TRP1, gal4, gal80*) transformed by a multi-copy plasmid bearing the reporter gene LexAop-LacZ. This eliminates the possibility that the interactions measured between the two components of the complex might be mediated by the third component. The activity of the LexA-Met28 fusion protein is stimulated 1000 fold by the presence of the Gal4-Met4 fusion protein, showing an interaction between Met4p and Met28p. To determine whether this interaction might involve the b-ZIP regions of these proteins, we used a Gal4-Met4 fusion protein lacking the

Met4p b-ZIP region. This fusion protein failed to stimulate the activity of the LexA-Met28 fusion. Conversely, no β-galactosidase acitivity is measured when the complete Gal4-Met4 fusion protein is expressed along with a LexA-Met28 fusion protein lacking the two last heptads of the C-terminal leucine zipper of Met28p. It has been shown that the expression of the carboxy-terminal domain of Cbf1p (amino acids from 208 to 351) restores the methionine auxotrophy of a *cbf1* disrupted mutant (11). We thus anticipated that this domain would be sufficient for the interaction with either Met4p or Met28p. Indeed, the activity of a LexA-Cbf1 protein (lacking the 200 first amino acids of Cbf1p) is stimulated 100 fold by the presence of a Gal4-Met4 fusion protein. In contrast, no stimulation is seen if we use a Gal4-Met4 fusion protein deleted from the Met4p leucine-zipper. We were not able to show a direct interaction betweenCbf1p and Met28p.

### Three new genes are involved in sulphate transport in *S. cerevisiae*

Using the genetic screen based on resistance to selenate, a toxic analogue of sulphate that allowed us to isolate *met28* mutants, we were able to isolate mutants impaired in sulphate uptake. These mutants were shown to bear mutations in three different complementation groups defining three genes that we called *SUL1*, *SUL2* and *SUL3*. Genetic and molecular studies have shown that genes *SUL1* and *SUL2* encode two high affinity sulphate transporters. The sequences of Sul1p and Sul2p indicate that they are integral membrane proteins exhibiting twelve transmembrane domains. In addition, these two proteins share a high degree of similarity. It is noteworthy that gene *SUL1* is identical to the already defined *SUL1* gene (16). Gene *SUL3* encodes a factor that is involved in the regulation of transcription of gene *SUL2* and is different from the regulatory factors described above (Cherest *et al*, Genetics 145, 627-635).

## Discussion

The yeast sulphate assimilation pathway can be considered as a model of a eukaryotic gene network. It comprises less than ten structural genes the expression of which is regulated in response to a single cellular signal: the increase of the intracellular concentration of AdoMet. According to this apparent simplicity, a simple picture has been drawn from the structure analysis of the 5' upstream regions of the sulphur genes. They contain one or two copies of a short upstream activating sequence containing the CACGTG core sequence and about ten adjacent nucleotides, and an upstream repressing sequence (URS MET) which is required for full repression of transcription (17, 6). In contrast to this simplicity, our work has revealed part of the complexity of the molecular mechanisms governing the regulation of the expression of this gene network, and which is summarized as a model in figure 7.

Three factors are involved in the transcription activation of the sulphur genes: two b-ZIP proteins, Met4p and Met28p, and one b-HLH-ZIP protein, Cbf1p (also involved in chromosomal segregation). Our results have shown that these three factors interact in a high molecular weight complex that binds to the promoter region of gene *MET16* chosen as a reporter gene of the network we study. In this complex, Met4p provides the transcription activation function.

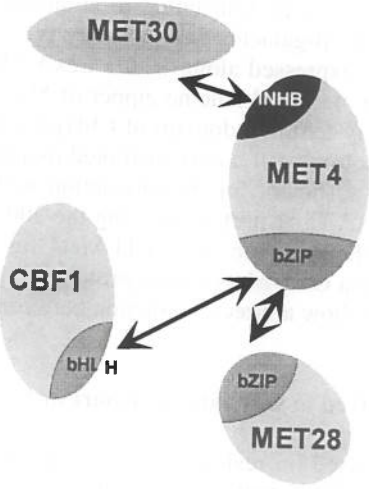


*Fig. 7.* Interactions between the different trans-acting factors regulating the transcription activation of the yeast sulphur genes.

Another protein, encoded by gene *MET30* has been shown to be the protein which inhibits the transcription activation function of Met4p in response to an increase of the intracellular concentration of AdoMet. Met30 belongs to the family of proteins bearing WD40 motifs and it has been shown that one motif is enough to interact specifically with another protein (5). The lethality associated with the disruption of the *MET30* gene is consistent with the involvement of Met30 in the regulation of other sets of genes than the sulphur genes. Indeed, we have shown that Met30 is part of the regulation of the genes involved in the methyl cycle (19) and in the pentose phosphate pathway (Thomas et al, unpublished results).

Preliminary experiments from this laboratory show that the assembly of the high molecular complex is regulated. The challenge for the future is to understand how it is regulated and how it is used by a eukaryotic organism to regulate in turn the expression of genes.

## Nucleotide sequence accession numbers

The sequence of the different genes cited have been assigned the following EMBL/GenBank accession numbers: *MET4*: M84455; *MET28*: U17015; *MET30*: L26505; *CBF1*: X52137; *SUL1*: X82013;

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# ASSIMILATORY REDUCTION OF INORGANIC SULPHATE

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## Abstract

Prototrophic organisms like bacteria, fungi and plants reduce inorganic sulphate to sulphide which is used for the biosynthesis of cysteine and methionine. This survey compares primary structures of the genes and enzymes involved in the process of reduction. It appears that the molecular basis of the gene products is conserved among the different organisms, thus confirming their general function in prototrophy. Higher plants are found to contain cDNAs encoding gene products that are homologous to the bacterial or fungal proteins. ATP-sulphurylase, APS-kinase and ferredoxin:sulphite reductase are highly conserved isofuntional enzymes. The gene structures also confirm the view that these proteins are predominantly localized in chloroplasts which can supply the necessary energy to drive the reduction. The reductive steps in higher plants differ from the reaction pathway found in bacteria and lower eukaryotes in that they use ferredoxin as reductant. Sulphite formation is also not yet fully understood and the function of a sulphonucleotide reductase which uses APS instead of PAPS remains to be elucidated. Altough conclusive evidence showing that these enzymes are responsible for sulphate assimilation in plant still is missing, they can be integrated into a tentative reaction scheme that replaces the bound sulphite as intermediate by free sulphite as in other prototrophic organisms.

## Introduction

Sulphur is an essential nutrient for all living organisms. In its divalent form sulphur occurs predominantly in the amino acids cysteine and methionine. Both amino acids are indispensable in maintaining the structure and function of proteins, redox-active cofactors, and coenzymes. Photoautotrophic plants, algae, cyanobacteria and a wide range of heterotrophic microrganism can use inorganic sulphate as their sole S. Where higher plants or algae accumulate secondary metabolites (86) or S-rich storage products (60) reduced or mixed forms of reduced and oxidized sulphur can occur in considerably higher concentrations. However, in many of these S-accumulating plants the role of these S-containing compounds is not always clear.

Physiological and biochemical aspects related to plant sulphate assimilation and its regulation in higher plants, algae and microorganisms have already been discussed most thoroughly in the excellent reviews of the first and second Sulphur Workshops (12, 80, 84). What little can be added to these earlier reviews is compiled from data obtained by methods of recombinant DNA technology (41, 105). However, biochemical aspects will also be included since one of the great advantages of this technique is that, in addition to the detection of homologous genes or iso-functional gene products, recombinant enzymes can be studied in homogeneous form. In this article, I will focus on the structure of genes, or cDNAs and their corresponding gene products, and attempt to outline their related properties with respect to different but species-specific features that underly the path of inorganic sulphate to sulphide in eubacteria, lower eukaryotes and higher plants. As a consequence of the molecular approach, this survey presents a slightly different view of the reaction sequence.

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**Structure of the DNA encoding sulphate reducing enzymes and *in vitro* properties of the gene products**

A molecular approach to sulphate assimilation in plants was hampered primarily by the lack of auxotrophic mutants and polypeptide sequence data that could have served as the basis for the construction of oligonucleotides. In addition to the unsettled questions about the flow of sulphate through the assimilatory pathway, the situation was complicated further by the discovery of cytosolic, plastidic and mitochondrial iso-enzymes (survey in 5, 53, 68). *In toto*, these deficits had to be overcome in order to establish a clear correlation between phenotype and gene. At the beginning of the search for genes, auxotrophic *cys* mutants from *Escherichia coli* or the homologous *MET* mutants from *Saccharomyces cerevisiae* proved extremely useful in the identification of cloned plant cDNA. Mutants that were succesfully complemented by a heterologous gene or cDNA were recognized by their ability to grow on inorganic sulphate. Thus, cDNAs encoding ATP-sulphurylase (35, 45), and *O*-acetylserine(thiol)lyase (72, 75, 116) were among the first higher plant genes that were isolated. The most recent example of sucessful complementation is the detection and identification of a cDNA encoding a PAPS-reductase homologue from *Arabidopsis thaliana* (see 24, 46) and from heterotrophic cell suspension cultures of *Catharanthus roseus* (110). A quite remarkable example of complementation was shown with a plant ATP-sulphurylase clone used to complement ATP-sulphurylase deficient *E. coli* (59). The enzymes from these organisms are not homologous or structurally related, yet the functional gene product from the higher plant, still including its chloroplast transit peptide, was sufficient to support prototrophic growth of the *E. coli* mutants *cys*D and *cys*N. Phenotypic complementation is, however, limited to gene products that lack species-specific cofactors, or auxiliary enzymes that assist in the biosynthesis of prosthetic groups, or in protein folding or post translational modification. Of the sulphate assimilating enzymes ferredoxin:sulphite reductase contains a sirohaem and an orthorhombic $Fe_4/S_4$ iron-sulphur cluster in its catalytic centre. The DNA of this enzyme was detected by heterologous hybridisation (22) in the cyanobacterium *Synechococcus* using a fragment of *cys*I from *E. coli* as DNA probe whereas the higher plant sulphite reductase and APS-kinase cDNAs were discovered by a PCR technique using highly degenerate primers (6, 10). In the case of sulphite reductase the primers were deduced from conserved domains of the polypeptide sequences of different sulphite- and nitrite reductases (23).

Based on genetic evidence, the path of reduction of inorganic sulphate to sulphide involves four enzymes in heterotrophic eubacteria and lower eukaryotes: ATP sulphurylase, APS-kinase, PAPS-reductase, and NADPH:sulphite-reductase. In photoautotrophic organisms like plants and cyanobacteria sulphite is reduced by a ferredoxin:sulphite-reductase and hydrogen sulphide formed in this reaction sequence is inserted into *O*-acetyl-serine by a thiol-lyase to give cysteine. The last step is different in *Saccharomyces cerevisiae* because this organism uses an *O*-acetylhomoserine(thiol)lyase yielding homocysteine.

*a. ATP-sulphurylase*

Sulphate activation is catalyzed by two enzymes: ATP-sulphurylase and APS-kinase. ATP-sulphurylase ($MgATP:SO_4^{2-}$ adenylyltransferase, EC 2.7.7.4) activates the relatively inert sulphate anion forming adenylyl sulphate (APS) as reaction product:

$$SO_4^{2-} + MgATP^{2-} \rightleftharpoons APS^{2-} + MgPPi^{2-}$$

Due to thermodynamic constraints ($K_{eq}$ $10^{-8}$, $\Delta G = -46$ kJ) and catalytic properties of the enzyme (inhibition by APS, $K_i < 0.04$ µM (97)) the rate of the forward reaction is negligible and the maximum level of APS that could accumulate under physiological concentrations of ATP (1 mM) and sulphate (1 to 10 mM) would range from 1 to 10 nM (14, 71, 97, 102, 114). The first reaction in the sequence of sulphate assimilating steps, however, is coupled to APS-kinase and to pyrophosphatase. APS-kinase with its high affinity for APS may help to prevent product inhibition of the sulphurylase reaction by binding the free APS formed. Together with inorganic pyrophosphatase hydrolysing the second reaction product of ATP-sulphurylase, kinase and phosphatase and sulphurylase allow 3'-phospho-adenylyl sulphate (PAPS) to accumulate.

Two independent, structurally not related families of ATP-sulphurylases have been found. In plants, fungi and a chemoautotroph sulphur oxidizer, ATP-sulphurylases are homo-oligomers formed from identical subunits whereas in enterobacteria and in rhizobia ATP-sulphurylases are composed of two different types of subunits.

ATP-sulphurylases in plants appear to form a family with three members of considerable structural identity (35, 45, 52). Their cDNAs (*sul, aps*) encode gene products ranging from 48 to 53.6 kDa where the larger size refers to gene products including the plastidic transit peptides and the smaller size seems to represent a cytosolic form. Isoforms have previously been discovered in spinach leaves (68). The spinach ATP-sulphurylases have a native MW of 170 kDa – with a subunit size of 49 kDa for the cytosolic and 50 kDa for the mature plastidic form suggesting a homo-tetramer for the active protein.

ATP-sulphurylases from fungi (*met*3, *aps, s*C) are structurally related to the plant sulphurylases. However, genes from the plectomycetes *Penicillium chrysogenum* (20) and *Aspergillus nidulans* (8) encode for larger proteins (64.3 kDa for *P. chrysogenum*). In comparison to plant ATP-sulphurylases, the gene products from *S. cerevisiae*, *P. chrysogenum* and *A. nidulans* have C-terminal extensions of 120, 172, or 161 amino acid residues. In the case of *P. chrysogenum* and *A. nidulans* these sequences have been described as APS-kinase like (8, 20). However, these larger proteins do not form PAPS. It appears that these extensions serve as a regulatory device responsible for allosteric control by PAPS that had been reported earlier for the enzyme from *P. chrysogenum* (20, 70) (figure 1).

The eubacterial ATP-sulphurylases are divided into two groups: one group similar to yeast or plant sulphurylases like the gene product found in the chemoautotrophic *Riftia* symbiont (44) and the other group represented by the heterodimeric sulphurylases originally found in *E. coli* (encoded by *cys*DN). The diazotrophic *Rhizobium meliloti* and *Azotobacter brasilense* belong to this third group. *R. meliloti* was reported to contain in addition to the two copies of *nod*PQ located on the megaplasmids pSymA and pSymB a third locus termed *saa* that may also encode ATP-sulphurylase and APS-kinase (89). Enterobacterial ATP-sulphurylases are heterodimeric proteins consisting of a small subunit (35 kDa encoded by *cys*D) and a large subunit (53 kDa encoded by *cys*N). An active recombinant ATP-sulphurylase is formed from both subunits. It is reported to catalyse APS-formation only when activated by GTP (50). Hydrolysis of GTP has been interpreted a mechanism to drive the formation of APS (51, 112) A close relationship between the genes *nod*P and *nod*Q and *cys*DN can be expected since *cys*DN from *E. coli* was used as probe to detect and identify the homologous DNA from *Rhizobia* (87, 88). The gene *Nod*Q like *s*C or *aps* from the filamentous fungi codes for an ATP-sulphurylase with an extension at the C-terminus which on the basis of deduced amino acids is homologous to APS-kinase (*cys*C or MET14, c.f. 8). Yet, in *Rhizobium meliloti* this extension is as catalytically active as APS-kinase when it associates with the *nod*P gene product forming a bifunctional sulphate activation complex that can be activated by GTP (90). On the basis of its amino acid sequence this complex is partially homologous to the bifunctional

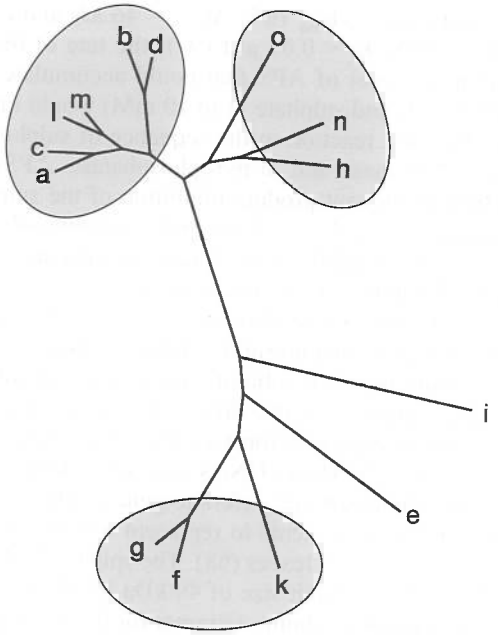


*Fig. 1.* Unrooted phylogenetic tree of ATP-sulphurylases showing the relative distance of homologous gene products from plants, fungi and animals. The highly related plant polypeptides from *Arabidopsis thaliana*: (a) accession no.: U59738, (b) U59737, (c) U05218, *Brassica oleracea*: (d) U69694, *Solanum tuberosum*: (l) S44267, (m) S44079 form a small family related to the independent groups of ATP-sulphurylases from fungi (*Emericella nidulans*: (f) X82541, *Penicillium chrysogenum*: (g) A53651, *Saccharomyces cerevisiae*: (k) P05836) and animals (*Mus musculus*: (h) U34883, *Urechis caupo*: (n) L39001, and *Caenorhabditis elegans* (o) Z68880). *Chlamydomonas reinhardii* (e) U57088 and the *Riftia* symbiont (i) L26897 are distantly related to the protein from fungi. ATP-sulphurylases from enterobacteria (*Escherichia coli cys*D gene product, accession no.: P21156, and *cys*N gene product P23845) and the iso-functional gene products from diazotrophs represent a structurally unrelated group that has not been included in the alignment using *Clustal* and *TreeGen*.

ATP-sulphurylase-APS-kinase from mouse brain (48, 54) or the marine worm *Urechis caupo* (73). The proteins from these two animals are a bifunctional ATP-sulphurylase – APS-kinase that are encoded by a single open reading frame. The term PAPS-synthetase was coined for the protein from *Urechis caupo*.

The apparent heterogeneity of the structure of ATP-sulphurylases seems to indicate that both families have evolved independently. It is noteworthy, however, that all ATP-suphurylases described today lack the typical ATP-binding domain. This ATP-binding domain is formed by a glycine rich loop, the so called A type motif (the Walker box: GxxxxGK, or P loop), a B motif consisting of the conserved amino acids DxxG and the C motif NKxD (38, 107). These ATP metabolising proteins contain a highly conserved hydrophobi1c β-strand with a turn into an α-helix (77) forming a loop. This structure is essential in hydrolysing the β-γ phosphate group of the NTP, yet except for the GTP activation site ($G_{34}$SVDDGK in *cys*N, $G_{31}$SVDDGK in *nod*Q), all the other ATP-sulphurylases lack the typical P-loop GxxxxGK. The absence of this A motif and also of the DxxG and the NKxD motif raises the question of whether ATP- sulphurylases belong to this ATP-superfamily at all. As pointed out by Koonin (38) ATP-sulphurylases cleave ATP at its α-β bond but not its β-γ phosphate. The latter type of cleavage is a typical reaction of adenyl transferases (39). In view of the diverse structural and catalytical

properties of adenyl transferases it has been suggested that these enzymes could not have evolved from a single phylogenetic origin (9). Thus, co-evolution may explain the occurrence of the two non-related ATP-sulphurylase families.

*b. APS-kinase*

APS-kinase (ATP:adenylyl sulphate 3'-phosphotransferase, EC 2.7.1.25) phosphorylates APS at the 3' position of the ribose moiety:

$$APS^{2-} + MgATP^{2-} \longrightarrow PAPS^{4-} + MgADP^{-}$$

With its high affinity (32,69,79) for APS the enzyme is readily saturated at submicromolar concentrations of substrate. A severe substrate inhibition occurs already in the micromolar range but this inhibition can be alleviated by high ionic strength (69) as is the case for APS-kinase from *P. chrysogenum* or by thioredoxins (92) as used for the enzymes from *E. coli* or *C. reinhardii*. In *E. coli* APS-kinase, the reaction mechanism has been shown to involve a phosphorylated enzyme intermediate and it seems that the rate of autophosphorylation limits the overall reaction (78, 79). Substrate channelling between ATP-sulphurylase and APS-kinase was reported for the mammalian bifunctional enzyme (54). Irrespective of the differences in the reaction mechanisms, the kinetic data unequivocally show that APS-kinase is extremely efficient with $V_{max}/K_{mAPS}$ ratios exceeding $10^8$ $M^{-1}$ $s^{-1}$ (79). As a consequence, PAPS is formed even at the very low the concentrations of APS provided by ATP-sulphurylase.

APS-kinases (encoded by *cys*C (47), *met*14 (40) *nod*Q (87) *akn* (6), *kin* (31)) form a very uniform group of small proteins with a molecular weight ranging from 22.3 kDa (*E. coli*) to 29.7 kDa (*A. thaliana* including its transit peptide). The APS-kinases show a high degree of identity over the entire length of the polypetide chain (figure 2). The highly conserved amino acid sequences (47.5 to 55.4% identity, including also APS-kinases from the bifunctional PAPS-synthetases) contain two of the three motifs that belong to the NTP-binding domain (i.e. motif A in TGLSGSGKST, and motif B in DPKGL (6)). According to Satishandran et *al.* (79), APS-kinase catalyses a phospho-transferase reaction with a phosphorylated enzyme intermediate. A serine residue was identified as transitory phosphoryl group acceptor. It is located in a L/FISP sequence between the two NTP binding motifs GxxxxGK and DxxG. If the reaction mechanism proposed for the kinase (79) is correct the γ phosphate group of ATP as well as to the 3'-OH group of the ribose of APS should have access to this serine. As phosphorylation by ATP of the serine in the reaction centre precedes transfer of the phosphoryl group to the 3'-OH group of the ribose, APS entering the catalytic site before ATP causes substrate inhibition. If there is any binding of the product PAPS, as speculated for the back reaction, it should bind to the same reaction site where APS is phosphorylated. In this respect it is noteworthy that a modified P-loop motif (GxxGxxK) was identified by site specific mutagenesis of PAPS-dependent sulphotransferases (15, 37) as the binding site for PAPS. With the exception of the APS-kinases from yeast and from *P. chrysogenum* where glycine is replaced by alanine, this motif occurs in all other APS-kinases and supports the proposal that GxxGxGKS represents a combined ATP/GTP and PAPS-binding site.

Metabolic control of the APS-kinase from *Chlamydomonas reinhardii* was observed to depend on a thiol-disulphide redox charge with reduced thioredoxin m as specific activator (92). This finding may be significant also for higher plants as the *Arabidopsis akn* gene product contains eight cysteines per subunit. Four cysteine residues are grouped in pairs flanking the NTP binding site and serine 182 in the putative catalytic centre. Cysteine(s)

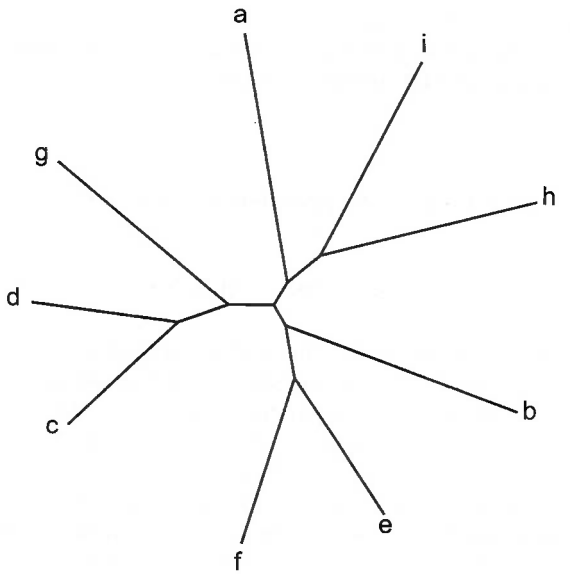


*Fig. 2.* Unrooted phylogenetic tree of APS-kinases showing the highly related members of the same family. The relative distances between the polypeptides from *Escherichia coli*: (a) U29579, *Synechocystis sp.*: (i) D90902, *Pseudomonas aeruginosa*: (h)P29811, *Arabidopsis thaliana*: (b) X75782, S47640, *Saccharomyces cerevisiae*: (e) X57990, *Penicillium chrysogenum*: (f) U39393, *Caenorhabditis elegans*: (g) Z68880, *Mus musculus*: (c) U34883, *Urechis caupo*: (d) L39001 are virtually identical. Of the PAPS-synthetases only the APS-kinase segment is used for alignment.

that could play a role in a redox mediated regulation of APS-kinase activity are not yet identified. The plant kinase forms stable dimers as does the yeast APS-kinase which also has a high content of cysteines. The enzyme from a mesophil *P. chrysogenum* is less stable; it monomerizes when the temperature is raised but reassociates into the active dimer at lower temperatures. Perhaps not surprisingly, this protein does not contain a single cysteine (20). The high content of cysteinyl residues in the plant enzyme may explain not only the stability of the dimer but also its side reaction as APS-sulphotransferase (6, 81).

Only a plastidic form of the protein has so far been identified (6, 31). This location would restrict biosynthesis of the activated sulphate PAPS to the chloroplast. Assuming that PAPS is not exported from the chloroplasts, this would imply that a cytosolic form of APS-kinase may exist, too, providing PAPS for the synthesis of sulphated secondary metabolites. However, a cytosolic APS-kinase may only occur in species that form glucosinolates (see Schnug, p. 109), flavonol- (2), tyrosin- (57, 74) or choline sulphates (26) and sulphated carbohydrates. Moreover, since many of these compounds are transported into the vacuole (see Mornet *et al.*, p. 1) or excreted, cytosolic APS-kinase may be attached to the tonoplast or the cytoplasmic membrane system.

*c. PAPS-reductase*

PAPS-reductase reduces PAPS to free sulphite and adenosine 3', 5'-bisphosphate (94) without formation of an intermediate of the RS:$SO_3H$ structure. Thioredoxin as reductant was originally detected by Porqué et *al.* (67) :

$$PAPS^{4-} + trx(SH)_2 \longrightarrow HSO_3^- + PAP^{4-} + H^+ + trx(S)_2$$

Homogeneous PAPS-reductase is specific for PAPS ($K_m$ 10 µM in *E. coli*, (7)) and will not reduce APS *in vitro*, but thioredoxin (7) as reductant can be replaced by glutaredoxin *in vivo* (108) and *in vitro* (Stoltze, Aslund, Schwenn, unpublished). The reaction product 3',5'-PAP is a potent inhibitor ($K_i$ 4 µM, (7,93)) but its level can be controlled by a 3'-nucleotidase (the *cys*Q or *met*22 gene product). As discussed in the contributions by Leustek and by Wray (pp. S & T) the (P)APS-reductase from higher plants preferentially uses APS over PAPS in a reaction that does not depent on exogeneous thioredoxin.

PAPS-reductases are encoded by *cys*H (*E. coli, cys*H' in *T. roseopersicina* (7, 42, 64)), *met*16 (*S. cerevisiae* (107), *s*A (*Emericella nidulans*, formerly *Aspergillus nidulans* (8), and *par* for *Synechococcus* (62). (P)APS-reductase from *Arabidopsis thaliana* has been designated APR (46, 96) or PRH (24) (pp, U, V.). We isolated most recently a cDNA (termed *par*) from a cell suspension culture of *Catharanthus roseus* encoding a sulphonucleotide reductase. These cells are heterotrophic and contain proplastids but the gene product of *par* is structurally very similar with the gene products APR or PRH from *A. thaliana*.

The PAPS-reductases encoded by bacterial or fungal genes form a family of loosely related proteins that can be divided into a eubacterial and a fungal subgroup (Figure:X). The binary matching coefficients ($S_{AB}$)[1] between *E. coli* PAPS-reductase and the enzyme from *Synechococcus* is 0.52, and 0.6 with the enzyme from *T. roseopersicina* (a photosynthetic purple sulphur bacterium). When *E. coli* is compared with *S. cerevisiae*, $S_{AB}$ is found to be 0.26 but among the fungi, comparing *S. cerevisiae* with with *S. pombe* or *Emericellus nidulans* it is 0.78. Unexpectedly, CysD gene product i.e. the small subunit of the *E. coli* ATP-sulphurylase, has a remarkable similarity to CysH PAPS-reductase (9).

The plant type (P)APS-reductases form an independent third group of gene products (figure 3). Corresponding cDNA clones encoding this protein were detected by phenotypic complementation of the *E. coli* PAPS-reductase mutant JM96 (24, 96) or by complementation of the yeast mutant MET16 (110). Complementation of the *E. coli* APS-kinase mutant JM81A indicates its capability of using APS as substrate. The gene products are larger (ranging from to 51.4 kDa in *C. roseus* to 50.4 in *A. thaliana*) due to the transit sequence at the N-terminus and an extension at their C-terminus that may be interpreted as thioredoxin-like polypeptide. Both plant sulphonucleotide reductases are highly homologous (82% identity). However, the identity of the core protein of *APR* with the amino acid sequence of the bacterial polypeptide cysH is very low: 26% identity, 48% similarity for *A. thaliana* and 28% identity, 55% similarity)[2] for the sulphonucleotide reductase from *C. roseus*. The evolutionary distance to the polypeptide from lower eukaryotes is similar. It appears that the C-terminus of the plant (P)APS-reductase, in comparison to the small PAPS-reductases from fungi, is extended further by a thioredoxin/protein disulphide isomerase-like polypeptide. The identity of this part of the (P)APS-reductase with the C-terminal part of the protein disulphide isomerase from chicken is 30%. (P)APS-reductases from *A. thaliana* (24, 96) and from *C. roseus* (110) contain a transit peptide at their N-terminus which is rich in hydrophilic, basic and hydroxylated residues. It is assumed that this transit peptide directs the plant (P)APS-reductase into the plastid where processing by a stromal protease would yield a mature protein of 44 kDa.

[1] $S_{AB}$, binary matching coefficient is the ratio of (number of amino acids in identical position in sequence A and B) x2 to (total number of amino acids in A) + (total number of amino acids in B).

[2] According to Dayhoff (17a) members of the same family show sequence identies of > 50 %. With its low sequence identity of < 30 % the par protein would not belong to the same superfamily.

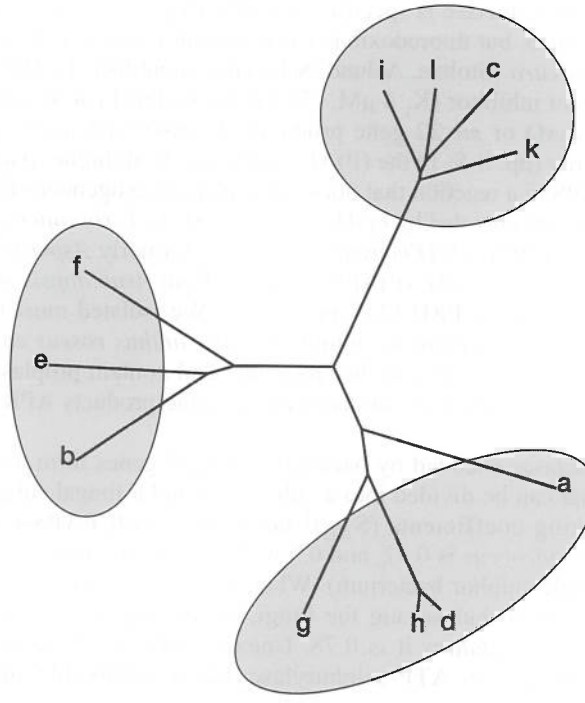


*Fig. 3.* Unrooted phylogenetic tree separating a small family of plant (P)APS reductases (*Catharanthus roseus*: (c) U63784, *Arabidopsis thaliana*: (i) U43412, (k) U56922 and (l) U56821) from the homologous gene products of bacteria (*Escherichia coli*: (d) P17854, *Salmonella typhimurium*: (h) P17853, *Synechococcus* PCC7942: (a) S28609, *Thiocapsa roseopersicina*: (g) S34193 and fungí (*Emericella nidulans*: (b) X82555, *Saccharomyces cerevisiae*: (e) P18408, *Schizosaccharomyces pombe*: (f) Z69729) polypeptides.

From biochemical work it is known that active PAPS-reductase forms a stable dimer. The molecular weights found under non-denaturing conditions range from 55.8 kDa for the enzyme from *E. coli*, (42), 66-68 kDa for the yeast (94,106) and 72-74 kDa for the spinach enzyme (95). Bacterial and fungal PAPS-reductases investigated so far contain a conserved cysteine located near the C-terminus but are devoid of typical chromophores that could store electrons required for reduction of PAPS. The enzyme from *E. coli* has been purified to homogeneity and the 3D structure of the reduced form has now been solved. The monomer forms a trough-like catalytic cleft lined by six parallel β-sheets. The β-sheets are connected by short α-helices that form the outer surface. The charge of the inner surface is highly positve presumably pulling the substrate with its four negative charges into the reaction site (Sinning & Schwenn, unpublished). From the current biochemical characterization it is assumed that the C-terminal sequence contains the catalytic centre ($K_{236}$xECGL/IH). It resides in the C-terminal α-helix which is highly flexible in the reduced form. In the oxidised dimer, these cysteine residues form a stable disulphide bridge. Difference spectroscopy of reduced versus oxidized protein from *E. coli* and site specific mutagenesis indicated that in addition to the thiol group of the intramolecular glutathione-like cysteine239, a tryptophan185 and a tyrosine209 may be involved in the charge transfer, too (7). It is not surprising that these essential residues are conserved in the higher plant PAPS-reductase. Moreover, short segments of identical amino acids can be found in $D_{144}$TGRLNPETY, $K_{252}$VR, $R_{258}$AL, $Q_{282}$GYVSIG. These con-

served amino acids are found in regions that are predicted to form a β-turn-sheet- or β-turn-sheet- β-turn structures (16)[3].

The motif K-$EC_{317}$GL/IH that is assumed to represent the reaction centre of the putative plant (P)APS-reductase is also conserved although it lacks the basic or hydrophilic amino acid between the lysine and the glutamate residue found in all the other PAPS-reductases. The K-ECGL/IH motif is flanked by two CxxC tetrapeptides: $C_{289}$EPC adjacent to the conserved $GY_{283}$ and further towards the C-terminus $C_{383}$RFC in the thioredoxin resembling segment. In view of the redox chemistry of the enzyme, it is tempting to speculate that the protein uses this part of the sequence as thioredoxin-like dithiol-disulphide redox couple.This speculation is supported by the recent finding that deletion of the C-terminal thioredoxin-like polypetide sequence of the *C. roseus* (P)APS-reductase yielded an inactive enzyme that could be restored by addition of thioredoxin (110). However, the properties of this cysteine tetra peptide in YAPW$C_{383}$RFCQ are not entirely thioredoxin-like because glutathione that can be used as reductant in the (P)APS-reductase assay does not reduce true thioredoxins (28, 29). Whatever the physiological reductant of this (P)APS-reductase may be, free ionic sulphite can be expected as product in the photosynthetically active chloroplast with its high negative redox potential formed by reduced glutathione (45). Taking the structural and biochemical properties of the new plant type (P)APS-reductases together, it appears unlikely that the enzyme follows a sulphotransferase mechanism. Hence, a trivial name like APS-reductase would be more appropriate. However, this term is assigned to the iron-sulphur flavoprotein adenylylsulphate reductase (EC 1.8.99.2) from dissimilatory sulphate reducers (103) and sulphide oxidisers (65). As long as we do not know exactly what the function of this protein is *in planta*, and in order to avoid confusion with the iron-sulphur flavoprotein, (P)APS-reductase may be useful as a description of a protein that is structurally homologous to trx:PAPS-reductases but preferentially uses APS as substrate.

*d. sulphite reductase*

Sulphide that is required for cysteine or methionine biosynthesis is formed from sulphite by assimilatory sulphite reductases without releasing intermediates. Heterotrophic organisms employ an NADPH: sulphite reductase (EC 1.8.1.2, hydrogen sulphide: NADP+ oxidoreductase)

$$HSO_3^- + 3\,[NADPH + H^+] \longrightarrow HS^- + 3\,NADP^+ + 3\,H_2O$$

while photo-autotrophic organisms like cyanobacteria, green algae and higher plants use a ferredoxin:sulphite-reductase (EC 1.8.7.1):

$$HSO_3^- + 6\,ferredoxin_{red} + 6\,H^+ \longrightarrow HS^- + 6\,ferredoxin_{ox} + 3\,H_2O$$

NADPH: sulphite reductases from enterobacteria are oligomers of eight flavoproteins and four $Fe_4/S_4$ haemoproteins. The flavoproteins (termed α-subunits) contain one FMN and one FAD per monomer of 66 kDa, while the haemoproteins (β-subunits) contain one sirohaem and one $Fe_4/S_4$ cluster per monomer of 64 kDa (99, 100). Six electrons are transported from NADPH via FAD, FMN to the $Fe_4/S_4$-cluster and sirohaem where the sulphite is bound (101, 104). The biochemical studies of Siegel and coworkers showed

[3] Numbering of the residues refers to the polypeptide sequence from *Catharanthus roseus*.

very clearly that the catalytic properties of the enzyme from *E. coli* are ideally suited to produce hydrogen sulphide under physiologically relevant conditions without formation of detectable intermediates (98). Though similar in its kinetic properties, the eukaryotic NADPH-sulphite reductase from *S. cerevisiae* differs considerably in its structure from the enterobacterial enzyme. It is an oligomeric flavo-haemoprotein exhibiting an $\alpha_2$ $\beta_2$ structure of 116 kDa and 167 kDa subunits (36). The molecular architecture of ferredoxin-sulphite reductases is much simpler. They lack the flavoprotein moiety but consist of the sirohaem iron-sulphur proteins only. These sulphite reductases exist primarily as dimers (β2, 63 kDa per subunit). The affinity of the spinach enzyme towards reduced ferredoxin and sulphite ($K_m$ 19 and 20 μM, respectively) is high enough to meet physiological concentrations. Plant sulphite reductase can also use reduced methylviologen (paraquat) as reductant instead of ferredoxin and will reduce nitrite to ammonia though at a lower efficiency.

Sulphite reductases have been cloned from assimilatory and dissimilatory sulphate reducers. Considering only assimilatory sulphite reductases three related groups can be proposed today: the enterobacterial type of an $\alpha_8$-$\beta_4$ NADPH: sulphite reductase encoded by *cys*J and *cys*I (63, 64), the $\alpha_2$-$\beta_2$ NADPH: sulphite reductase of the lower eukaryotes as in yeast encoded by *met*10 and *met*5 (21, 25), and the ferredoxin-dependent haemoproteins of the photoautotrophic organisms encoded by *sir* from *Synechococcus* PCC7942 (23) and from *A. thaliana* (10) (figure 4). The flavoproteins encoded by *cys*J in *E. coli* or *S. typhimurium* and its homologue from the phototrophic purple sulphur bacterium *T. roseopersicina* each display a typical FAD and FMN binding site. These proteins share highly conserved segments with other dinucleotide binding flavoproteins, notably with the ferredoxin:NADP oxidoreductase (EC 1.18.1.2). The 3D structure of this protein is known (13, 34) and shows five motifs (I – V) that are required for the binding of FAD and NADP (figure 5a). Amino acids involved in the binding of the two cofactors are also conserved in the β-subunit of the NADPH-sulphite reductase. Among the flavo-enzymes with similar motifs are NADH-nitrate reductase from *A. thaliana*, NADH-cytochrome b5 reductase and NADPH-cytochrome P450 reductase. Ostrowski et al. (63) interpreted amino acid sequence identities between flavodoxin from *D. vulgaris* (113) and the first half of the flavoprotein, namely the two motifs $S_{70}$QTGNA and $F_{148}$SLGDTSYEFFC, as the possible binding sites for FMN. The first motif was assumed to bind the ribityl phosphate whereas the second may interact via hydrogen bonds with the pyrimidine moiety of the FMN isoalloxazine ring. The two domain structure of the flavoprotein with the N-terminal part of the sequence binding FMN and the C-terminal binding FAD and NADP was confirmed more recently using purified recombinant protein from *E. coli*. It was observed that the purified recombinant flavoprotein undergoes autocatalytic proteolysis to yield a 43 kDa C-terminal polypeptide binding FAD and an N-terminal 23 kDa fragment binding the FMN (18, 19). The flavoprotein from *S. cerevisiae* is a 115 kDa polypeptide encoded by *met*10 (25). Whilst only the carboxy-terminal sequence of approximately 400 residues is homologous with the β-subunit of the NADPH-sulphite reductase from enterobacteria, all the residues required for FAD and NADP binding are preserved in this part of the protein. The occurrence of an FMN-binding motif as found in *E. coli* flavoprotein could not be confirmed for the MET10 polypeptide. No homologue of this flavoprotein has yet been found in plants or algae.

The haem protein of sulphite reductases is encoded by *cys*I (64), *met*5 (21) or *sir* (10, 23). It contains an iron-sulphur cluster and sirohaem as prosthetic groups. A comparison of the deduced amino acid sequences showed a high degree of identity between the β-subunits of NADPH-dependent enzymes (from *E. coli* and *T. roseopersicina*, 60.4% identity) as the bacterial group and the ferredoxin-dependent enzymes (*Synechococcus, A. thaliana* 50.3% identity) as the plant group. The haem proteins of sulphite reductase

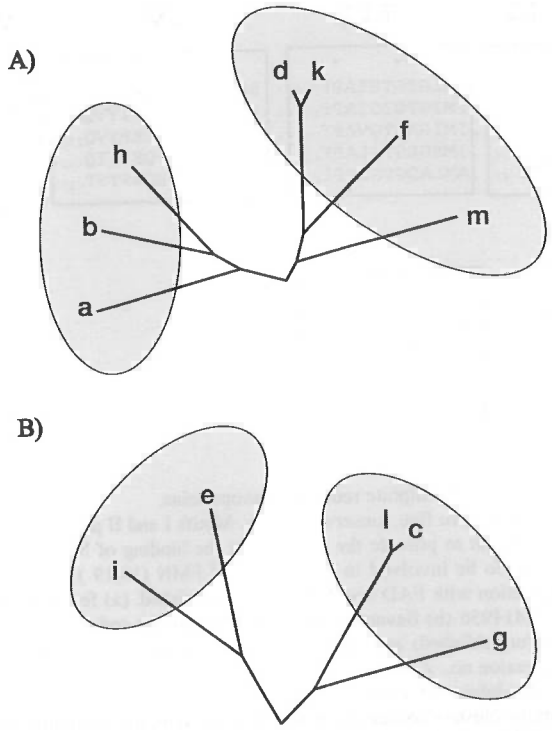


*Fig. 4.* A:Unrooted phylogenetic tree comparing the haem proteins of ferredoxin dependent sulphite reductases from *Arabidopsis thaliana*: (a) Z49217, *Synechococcus* PCC7942: (b) Z11755, *Synechocystis sp.*: (h) D90901 with the corresponding haem proteins from NADPH-dependent sulphite reductases from *Escherichia coli*: (d) M23008, *Salmonella typhimurium*: (k) M23007, *Thiocapsa roseopersicina*: (g) S34191 and *Saccharomyces cerevisiae*: (m) L26503.
B: Comparison of the flavin containing α-subunits of NADPH-dependent sulphite reductases: *Escherichia coli*: (d) M23008, *Salmonella typhimurium*: (k) M23007, *Thiocapsa roseopersicina*: (g) S34191, *Saccharomyces cerevisiae*: (m) L26503 and *Saccharomyces pasteurianus*: (i) L26504.

are comparable in size, ranging from 64 kDa for the bacterial NADPH-dependent enzyme to 65 kDa for the plant and 70 kDa for the cyanobacterial ferredoxin-dependent enzyme. The size of the *met*5 gene product is not yet known. Its coding sequence is contained in a large open reading frame of 1433 amino acids with an estimated weight of 166 kDa. A large β-subunit of 167 kDa was previously reported for the purified enzyme (36). It is noteworthy that only 610 amino acids of the carboxyterminal part are homologous to the β-subunits of other sulphite reductases.

A high resolution crystallographic structure of the *E. coli* haem protein of the sulphite reductase revealed that the sirohaem together with the ortho-rhombic $Fe_4/S_4$ cluster and the substrate anion is juxtaposed at the interphase of a β-α mixed-sheet domain (17). A cysteine thiolate (cys483) couples both cofactors by bridging Fe of the sirohaem with Fe of the $Fe_4/S_4$-cluster, confirming very clearly earlier data obtained by biochemical and biophysical studies (55, 58). The iron-sulphur cluster is bound by two pairs of cysteine thiolates: $C_{434}$VSFPTC and TG$C_{479}$PNGC. The cysteine motif is conserved in NADPH and ferredoxin dependent sulphite reductases from *E. coli, T. roseopersicina, Synechococcus, S. cerevisiae*, and *A. thaliana*. (figure 5b). In addition to these clustered identities, ten of

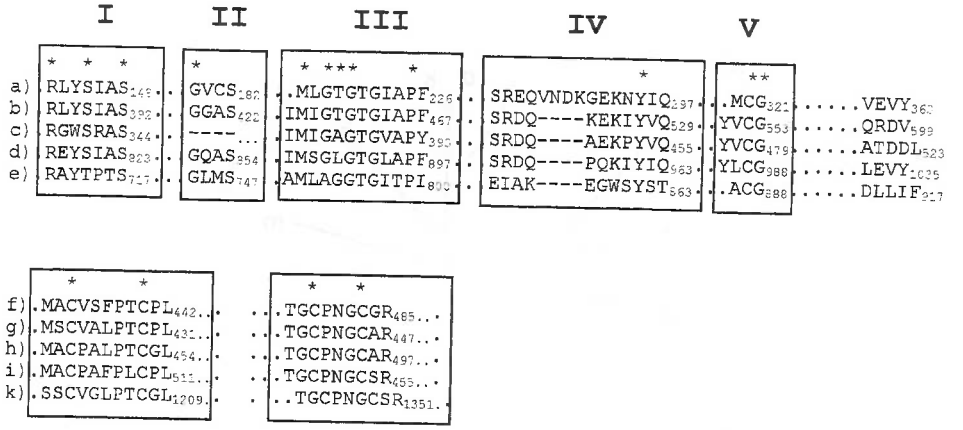


*Fig. 5.*
A: Cofactor binding sites of NADPH-sulphite reductase flavoproteins.
NADPH and FAD binding sites have five conserved motifs. Motifs I and II presumably interact with the FAD while motifs III to V are thought to provide the scaffold for the binding of NADPH. The N-terminus of the polypeptide that is proposed to be involved in the binding of FMN (18,19 ) is not shown; amino residues involved in the direct interaction with FAD and NADPH are asterisked. (a) ferredoxin-NADP reductase from *Vicia faba*, accession no.: M14956 (b) flavoprotein of NADPH-sulphite reductase from *E. coli* (63), (c) from *Thiocapsa roseopersicina* (unpublished) and (d) from *S. cerevisiae* (25), (e) flavoprotein of nitrate reductase from *A. thaliana* (115), accession no.: Z19050.
B: Cofactor binding sites of sulphite reductase haem proteins.
Alignment of the two cysteine clusters conserved in NADPH- and ferredoxin-sulphite reductase haem proteins. Cys483 is proposed to couple the Fe-haem center with the $Fe_4/S_4$ cluster. (f) haem protein from *E. coli*, (g) from *Thiocapsa roseopersicina*, ferredoxin-sulphite reductase from *Synechococcus* PCC7942( 23), *A. thaliana* (10), and β-subunit of the NADPH-sulphite reductase from *S. cerevisiae* (21).

fifteen amino acids (i.e.R85, R113, R117, R214, R485, Q120, Q396, K215, K217, T115, T439, S158, L157, and V435) involved as ligands for the acidic side chains of the siro-haem, are conserved in the ferredoxin-sulphite reductase. Perhaps most striking is that the 3D structures of ferredoxin-sulphite and of ferredoxin-nitrite reductase from *Synechococcus* are very similar (17). This finding was hardly predictable from a binary matching coefficient of 0.23.

Sulphide formed by the sulphite reductases is incorporated into *O*-acetyl-L-serine yielding cysteine in bacteria and plants but not in *S. cerevisiae*. The *O*-acetyl-L-serine-(thiol)-lyases are treated by Saito in this volume, p. 253.

## Bound intermediates in sulphate reduction by plants

Until now the established path of sulphur from sulphate to sulphide (and cysteine or methionine) in plants has been characterised by the formation of protein-bound intermediates rather than free ionic products such as sulphite and sulphide. The first reaction of the plant specific sulphate reduction is catalyzed by APS-sulphotransferase (80, 84):

$$APS^{2-} + CarSH \longrightarrow CarS{:}SO_3^- + AMP^{2-} + H^+$$

The enzymatic reaction, originally proposed by Schmidt (82) and Abrams and Schiff (1), requires a carrier thiol (designated carSH, XSH) of still unknown identity. Using a homogeneous APS-sulphotransferase from *Euglena gracilis* (49) the role of the carrier in the transfer mechanism has been refined as a carrier that accepts, in the form of a thiolate, a sulpho group from an intermediate enzyme-cysS:$SO_3H$ complex:

$$APS + E\text{-}cysS^- \longrightarrow APSxE\text{-}cysS^- \longrightarrow E\text{-}cysS{:}SO_3^- + AMP$$

$$E\text{-}cysS{:}SO_3^- + carS^- \longrightarrow carS{:}SO_3^- + E\text{-}cysS^-$$

The enzyme product complex can give rise to sulphite via exchange with free sulphite or via reductive cleavage by vicinal dithiols or excess monothiols:

$$E\text{-}cysS{:}{}^{35}SO_3^- + SO_3^{2-} \longleftrightarrow E\text{-}cysS{:}SO_3^- + {}^{35}SO_3^{2-}$$

$$E\text{-}cysS{:}SO_3^- + 2\ RSH \longleftrightarrow EcysS^- + HSO_3^- + H^+ + RS{:}SR.$$

Homogeneous APS-sulphotransferase from *Euglena gracilis* forms a tetramer of 102 kDa (subunit size 25 kDa) presumably held together by disulphide bonds. Its affinity towards APS is 0.1 µM when DTT is used as reductant. However, in the presence of thiols (30mM (49)) the enzyme is progressively inactivated giving rise to non-linear reaction kinetics. The biochemical properties of APS-sulphotransferase from higher plants described in the older literature (27, 63, 83) are difficult to assess because these measurements were made with cell extracts or partially enriched proteins. The major obstacle was a lack of stability during purification. Moreover, as the reaction is measured under reaction conditions (detailed in ref. 12) that are far from physiologically relevant and recombinant APS-kinase from *A. thaliana* expressed in *E. coli* was observed to catalyse an APS-sulphotransferase activity as side reaction (6, 81, 91), the significance of APS-sulphotransferase activity was questioned. Conflicting results such as these may be seen as indication of an inadequacy of the assay systems. It appears that different proteins have been isolated and assigned to APS-sulphotransferase. A re-examination of the size of APS-sulphotransferase from different organisms showed differences ranging from 330 kDa (in *Chlorella* (27)) to 110 kDa in spinach (63). Moreover, from heterotrophic cell suspension cultures a large complex of 700 kDa was obtained that contained APS-reducing activity together with disulphide reductase and ferredoxin:sulphite reductase. In contrast to the afore-mentioned proteins, the complex was measured as the formation of cysteine from APS in the presence of *O*-acetyl-L-serine-(thiol)-lyase and ferredoxin:NADPH oxidoreductase (111). More recently, a smaller enzyme exhibiting APS-sulphotransferase activity was isolated from *Porphyra yezoensis* (33) measuring the formation of AMP from APS in the presence of DTT. The native weight reported for the homogeneous protein was 350 kDa, resembling the enzyme from *Chlorella*. The authors assume that the *Porphyra* APS-sulphotransferase is composed of eight identical subunits of 43 kDa. Ara & Sekiya (pp.O) reported that APS-sulphotransferase from spinach could be purified extensively using a coupled assay system similar to ref. 111. In view of the new data obtained with the recombinant (P)APS-reductase it is desirable to analyse the polypeptide sequence of any homogeneous APS-sulphotransferase preparation and to see whether it is identical with the new (P)APS-reductase.

The second reaction of this bound-sulphite pathway is catalysed by a thiosulphonate-reductase. This enzyme was proposed to reduce bound sulphite to bound sulphide:

$$RS{:}SO_3H + 6\ ferredoxin_{red} + 6\ H^+ \longrightarrow RS{:}SH + 6\ ferredoxin_{ox} + 3\ H_2O$$

However, earlier reports of its occurrence in plants or algae have not been confirmed and further, under the conditions stated the reaction could have been catalysed by a sulphite reductase (98) acting upon free sulphite. Since ferredoxin:sulphite reductase is located in the plastid (4, 85), any free sulphite formed by a (P)APS-reductase or APS-sulpho-transferase may well be reduced further to sulphide. It must be kept in mind that sulphite is a strong reductant ($\Delta E_0$' -517 mV) and if formed in the plastid under oxidizing conditions it initiates a powerful radical reaction (3). Free sulphite also reacts spontaneously with protein disulphides or glutathione (GSSG $\Delta E_0$' -260 mV) to give S:sulpho-protein compounds or S:sulphoglutathione ($\Delta E_0$' – 402 mV)[4].

Sulphite formation in *E. coli* and *S. cerevisiae* is controlled through feedback inhibition of PAPS-reductase by 3',5'-PAP (7). This nucleotide is metabolised further by a 3'-nucleotidase to give AMP. 3',5'-adenosine-bisphosphate phosphohydrolase (EC 3.1.3.7) is encoded by the *cys*Q gene in *E. coli* (61). The mutant phenotype was detected only under aerobic growth. Although the growth defect mutant of *cys*Q can be compensated by supplying sulphite or cysteine its function is connected with a control of the intracellular level of PAPS, or its use in sulphite formation.

A cDNA clone with homology to this gene has recently been discovered in *Orizya sativa* by complementation of the corresponding mutants *cys*Q from *E. coli* and *met*22 from *S. cerevisiae* (66). The plant gene product has a molecular weight of 40 kDa and is structurally related to the family of inositol phosphatases. It hydrolyses PAPS, 3',5'-PAP and 2',5-PAP but not 3'-AMP. The authors suggested that this 3'(2)',5'-adenosine-bisphosphate phosphohydrolase could regulate the flux of sulphur by converting PAPS to APS. A futile cycle originally proposed by Tsang and Schiff (109) could then switch between sulphate reduction and sulphate conjugation. However, cDNA of the bisphosphatase from *O. sativa* encodes a cytosolic protein and it may well be that the physiological role of this bisphosphatase is to remove the 3',5'-PAP produced in PAPS-dependent sulphate ester formation. If APS is the only form of activated sulphate metabolised in the reduction process as proposed for the *A. thaliana* (P)APS-reductase (96), it would be necessary to include a plastidic PAPS-specific 3'-phosphohydrolase. This type of enzyme would also be required to remove 3',5'-PAP if PAPS is used by a *bona fide* PAPS-reductase (95).

## Conclusions

The molecular basis of enzymes involved in the assimilatory reduction of inorganic sulphate seems to be conserved among photosynthetic organisms, lower eukaryotes and prototrophic bacteria. There are, however, modifications in the structure of individual enzymes that appear to reflect specific adaptations of the organism as, for instance, the use of ferredoxin in plants in comparison to the NAD(P)H in heterotrophic bacteria or fungi. In view of the phylogenetic origin of chloroplasts it is perhaps not surprising that plants contain a set of sulphate reducing enzymes that is remarkably homologous to enzymes found in autotrophic prokaryotes.

It is obvious that the data as summarised are far from being complete. We have started to identify cDNA clones from libraries which are constructed from polyA RNA. As these libraries contain copies of only a fraction of the total RNA, they represent not more

[4] The oxidation of sulphite by GSSG is exergonic by -27.4 kJ/mol because the difference of redox potentials between GSSG/2GSH as oxidant and $GSSO_3H$/GSH + $SO_3^{2-}$ as reductant equals +142 mV (under standard conditions at 25°C).

than a fraction of the genome. The picture is also incomplete where multiple forms of a particular gene product have been found in the same organism. Consequently, the next step will be to find out the pattern of their regulation on the DNA and on the protein level. It is also obvious that they respond to external stimuli, and may alter their level in the course of development or due to production of specific secondary metabolites. The function of plastidic, cytosolic or mitochondrial isoforms may well be deduced from the recombinant gene product *in vitro*, but future work will also have to prove the function *in planta*, address the cellular distribution and control of enzymes operating in different compartments or organs. The first steps have been made in this direction by using the intact transgenic plant (76, 116) to study the activity or function of a particular gene. This type of investigation may become increasingly difficult when turning from the relatively simple genome of *Arabidopsis thaliana* to crop plants or even trees. Already by looking at the regulatory network that a lower eukaryote like *Neurospora crassa* (56) or *S. cerevisiae* (105) (Thomas *et al.*, p. 27) uses to control its S-metabolism one might anticipate what remains to be done to understand sulphur assimilation in higher plants.

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# MOLECULAR APPROACHES TO GLUTATHIONE BIOSYNTHESIS

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## Abstract

The thiol tripeptide glutathione is synthesized in plants from the constituent amino acids in two enzyme catalyzed reaction steps. The properties and the regulation of both enzymes, namely γ-glutamyl-cysteine synthetase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3), have been analyzed by classical biochemical studies and, recently, by plant molecular biology techniques. In the present review, the results achieved by these approaches are compared and a preliminary scheme for the regulation of cytosolic glutathione levels in plants is suggested with special emphasis on the interaction between the pathways of assimilatory sulphate reduction and glutathione biosynthesis.

## Introduction

In 1888 de Rey-Pailhade found that yeast and other cells contain a compound that spontaneously reacts with elemental sulphur to yield hydrogen sulphide. From the Greek words for love and sulphur he chose the name "philothion" for his substance (see: 22). Subsequent studies by Hopkins led to the conclusion that this compound is a dipeptide containing cysteine and glutamate; γ-glutamylcysteine was considered the most probable structure (see: 22). The name "glutathione" was chosen by Hopkins, because of its link with the historic philothion, the termination of "peptone" as a name for simpler peptides, and a reminder that it contains glutamic acid linked to a sulphur compound. A reinvestigation of Hopkins in 1929 led to the finding that the presence of glycine was overlooked in his former work and in 1930 the structure of glutathione was recognized as L-γ-glutamyl-L-cysteinyl-glycine (see: 22). This structure was frequently confirmed in later studies by chemical and enzymatic synthesis, degradation, and physical methods.

Today, glutathione is thought to be the most abundant compound within a group of thiol-containing γ-glutamyl-tripeptides, including in addition to glutathione, γ-glu-cys-ß-ala (phaseothione or homo-glutathione in the *Fabales*), γ-glu-cys-ser (hydroxymethyl-glutathione in the *Poaceae*), and γ-glu-cys-glu (in *Zea mays*) (see: 3, 15, 16, 24). These tripeptides may be present in cells either free, or as derivatives (e.g., of spermidine in *E. coli*, and of xenobiotics in bacteria, plants and animals) (18, 35, 42, 46) and "polymerization products" (e.g., the phytochelatins $(\gamma\text{-glu-cys})_n$gly, $(\gamma\text{-glu-cys})_n$ß-ala and $(\gamma\text{-glu-cys})_n$ser in plants) (17, 25, 28, 44). Glutathione and/or its analoguous γ-glutamyl-tripeptides are found in most procaryote and virtually all eucaryote cells (see: 35). They are generally considered products of primary metabolism not essential for life, but important for the detoxification of compounds unfavorable for growth (35).

Despite intensive studies with animal tissues (23) glutathione metabolism and function(s) in higher plants have not received considerable attention until the late 1980s. At this time it was recognized that glutathione can be an important factor in the defence of

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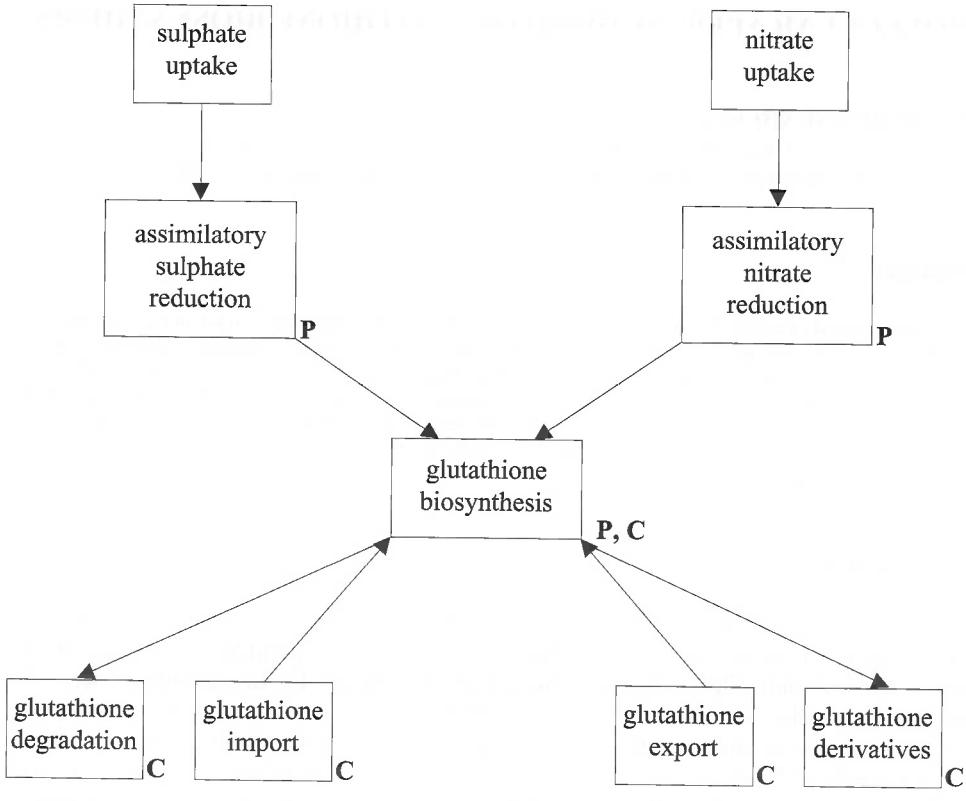


*Fig. 1.* Processes involved in the modulation of the glutathione concentration in plant cells and cellular compartments. Subcellular localization: P, in plastids; C, in the cytosol.

plants against various forms of stress, including mineral deficiencies, high light intensities, heat, cold, drought, heavy metals, xenobiotics, and pathogen attack (2, 32, 43). Today this function of glutathione partially has to be re-evaluated, since other compounds, e.g. ascorbate, can be more important as chemical antioxidants than glutathione (27, 36) and other reactions can contribute to the regeneration of reduced ascorbate than those of the classical Foyer-Halliwell pathway (27). Recent studies indicate that glutathione also plays an essential role in the regulation of sulphur nutrition in plants. Its long-distance transport mediates distribution of reduced sulphur within the plant to meet the sulphur requirements of individual organs in growth and development and, at the same time, can control sulphate influx at the level of uptake and xylem loading in the roots (13, 31, 33, 34, 41). From this dual set of functions of glutathione it appears that the actual glutathione concentration of plant tissue is an important factor in strategies that determine the flux of metabolites between growth on one side, and stress compensation and defence on the other (32).

The concentration of glutathione in plant tissues, plant cells and cellular compartments is the result of a number of interacting processes. These processes include (1) sulphate and nitrate uptake plus (2) assimilatory sulphate and nitrate reduction that provide metabolic precursors of glutathione, (3) regulation of the pathway of glutathione biosynthesis, (4) regulation of the pathway of glutathione degradation, (5) import and export of glutathione from xylem and phloem long-distance transport conduits, including xylem/phloem

unloading/loading and long-distance transport itself, and (6) glutathione turnover in the production of glutathione derivatives (Fig. 1). In this report, the present knowledge of the pathway of glutathione biosynthesis and its regulation in plants is summarized with special emphasis on recent studies applying techniques of plant molecular biology.

## Properties of the enzymes of glutathione biosynthesis

In plants, glutathione is synthetized from the constituent amino acids in two enzyme catalyzed reaction steps (Fig. 2), as also observed in bacterial and animal cells (23). In the first reaction a peptide bond is generated between the γ-carboxy-group of glutamate and the amino group of cysteine to yield γ-glutamylcysteine This reaction is ATP-dependent and is catalyzed by γ-glutamylcysteine synthetase (EC 6.3.2.2). In the second reaction the carboxy-group of cysteine in γ-glutamylcysteine serves as an acceptor of the amino group of glycine. The generation of the peptide bond between cysteine and glycine is catalyzed by glutathione synthetase (EC 6.3.2.3) at the expense of ATP hydrolysis and yields glutathione.

$$\text{glu} + \text{cys} + \text{ATP} + H_2O \xrightarrow{\gamma\text{-glutamylcysteine synthetase}} \gamma\text{-glu-cys} + \text{ADP} + P_i$$

$$\gamma\text{-glu-cys} + \text{gly} + \text{ATP} + H_2O \xrightarrow{\text{glutathione synthetase}} \gamma\text{-glu-cys-gly} + \text{ADP} + P_i$$

Both enzymes have recently been analyzed from plant sources both, in biochemical and molecular approaches.

### *γ-Glutamylcysteine synthetase*

Plant γ-glutamylcysteine synthetase is an extremely labile enzyme. It is readily inactivated by thiol protecting reagents such as dethioerythritol (DTE) or mercaptoethanol and requires analysis under strictly anaerobic conditions (12, 39). Since γ-glutamylcysteine synthetase activity is found in the cytosol and in the chloroplasts (3) and differences between cytosolic and chloroplastic isoforms can not be excluded, present data on the properties of the enzyme determined in cell homogenates can only be taken as an overall estimate.

As summarized in Table 1, plant γ-glutamylcysteine synthetase has a slightly alkaline pH maximum of 8.0 that is typical for many chloroplastic enzymes. It has an absolute requirement for $Mg^{++}$, as may be expected for an enzyme catalyzing an ATP-dependent reaction. The enzyme exhibits high affinity for cysteine and much lower affinity for glutamate reflecting the difference in cellular concentrations of these amino acids (3). Since cellular cys levels are frequently found to be lower than the apparent $k_M$ of γ-glutamylcysteine synthetase for cys, it appears feasible that *in vivo* cys availability can limit γ-glutamylcysteine synthesis by the enzyme. *In vitro*, γ-glutamylcysteine synthetase activity is inhibited by reduced glutathione (GSH) at concentrations frequently found in cell homogenates (3). Therefore, the rate of glutathione synthesis is thought to be regulated in plant cells by feedback inhibition of γ-glutamylcysteine synthetase by GSH (3). This feedback inhibition by GSH was found to be competitive with respect to glu (39). As also observed for γ-glutamylcysteine synthetase from mammalian sources, the plant enzyme is inhibited irreversibly by sulfoximine analogs of γ-glutamyl-phosphate, e.g., buthionine sulfoximine

(BSO) and L-methionine sulfoximine (MSO) (12, 23). Therefore, the formation of an enzyme-bound γ-glutamyl-phosphate that reacts with the amino group of cysteine also seems to be involved in the mechanism of the reaction catalyzed by plant γ-glutamylcysteine synthetase. From gel filtration a relative molecular mass ($M_r$) of the enzyme of 60 kDa was estimated. Since treatment with DTE results in a dissociation of the protein into identical subunits of 34 kDa $M_r$ accompanied by a heavy loss of activity, it appears that the enzyme is a homodimer with the subunits being connected via disulfide bonds that are involved in the determination of the structure of the catalytic centre of the enzyme.

Recently, a γ-glutamylcysteine synthetase cDNA of *Arabidopsis thaliana* was isolated by functional complementation of an *E. coli* mutant deficient in the enzyme and unable to synthetize glutathione (20). The cDNA isolated in this study encoded a single polypeptide of 60 kDa $M_r$, similar to the *E. coli*, but different from the tobacco enzyme (Table 1). The corresponding gene was expressed in leaves and roots and was present in a low number of copies (20). When compared with the published sequences of the genes of the rat, human, and *E. coli* enzyme, *Arabidopsis* γ-glutamylcysteine synthetase revealed only 15 to 18% identity. In addition, the *Arabidopsis* γ-glutamylcysteine synthetase cDNA probe failed to hybridize to maize and tobacco genomic DNA (20). From these observations the authors concluded a high degree of heterogeneity in the enzyme structure between different organisms. However, additional experiments are required to unequivocally prove that the cDNA isolated from *Arabidopsis* definitely encodes γ-glutamylcysteine synthetase. The recombinant *E. coli* clone, resulting from functional complementation of the *E. coli* mutant deficient in γ-glutamylcysteine synthetase, showed only 10% of the thiol level of the wild-type, despite high levels of extractable enzyme activity. Unfortunately, extractable γ-glutamylcysteine synthetase activity was determined from $P_i$ production by ATP hydrolysis in the assay mixture. This assay system is highly susceptible to interactions with numerous other reactions and cannot be used as a reliable measure of the activity of the enzyme, especially if the production of γ-glutamylcysteine in the assay mixture is not proven.

*Table 1.* Properties of γ-glutamylcysteine synthetase from higher plants sources (*Nicotiana tabacum, Petroselinum crispum, Arabidopsis thaliana*)*

| Biochemical studies | Molecular approaches |
|---|---|
| optimum pH: 8.0 | |
| absolute $Mg^{++}$ requirement | |
| $M_r$: 60 kDa | |
| homodimer | |
| subunit $M_r$: 34 kDa | $M_r$: 60 kDa |
| $k_M$ glu: 4.0 – 10.4 mM | |
| $k_M$ cys: 0.19 – 0.07 mM | |
| $k_I$ GSH: 0.27 – 0.42 mM | |
| BSO, MSO sensitivity | |
| activity in leaves and roots | expression in leaves and roots |
| activity in chloroplasts and the cytosol | |
| | mRNA size: 2 kb |
| | few gene copies |
| | poor homology to other eucaryotic and procaryotic sequences |

* data compiled from references 3, 12, 20, 39

*Glutathione synthetase*

Glutathione synthetase activity has been demonstrated in several plant species. Similar to γ-glutamylcysteine synthetase the enzyme was found in the cytosol, and in chloroplasts and proplastids as well (3). As mentioned above for γ-glutamylcysteine synthetase, cell homogenates also were used for the analysis of properties of glutathione synthetase and, therefore, the results achieved (Table 2) may represent a mixture of cytosolic and chloroplastic isoforms.

A slightly alkaline pH maximum, typical of chloroplastic enzymes, and an absolute $Mg^{++}$ requirement, as may be expected for an enzyme catalyzing an ATP-dependent reaction, was not only observed for γ-glutamylcysteine synthetase (Table 1), but also for glutathione synthetase (Table 2) from plant sources. In addition, plant glutathione synthetase is slightly stimulated by $K^+$ (3). The enzyme is characterized by a high affinity for γ-glutamylcysteine and an one order of magnitude lower affinity for gly, as may be expected from the difference in cellular concentrations of these substrates. Cellular γ-glutamylcysteine concentrations may be close to, or somewhat lower than the apparent $k_M$ of glutathione synthetase for γ-glutamylcysteine and, thus, may be a limiting factor in glutathione biosynthesis. Glutathione synthetase seems to be highly specific for gly and does not use ß-ala as a substrate. This and other observations have led to the conclusion that homo-glutathione (γ-glu-cys-ß-ala) is synthetized by a ß-ala-specific homo-glutathione synthetase (3). Evidence for a ser-specific "glutathione synthetase" that may catalyze hydroxymethyl-glutathione (γ-glu-cys-ser) biosynthesis has so far not been reported.

Plant glutathione synthetase is a homodimer of 113 – 120 kDa $M_r$ (3) and, thus, is similar to the enzyme from other eukaryotic sources (14, 21, 47). Gel filtration revealed a subunit size of 68 kDa $M_r$ (11). By contrast, the *E. coli* enzyme is a homotetramer with a subunit size of 38 kDa $M_r$ (10). Recently, *E. coli* and yeast mutants deficient in glutathione synthetase were used to isolate glutathione synthetase cDNA from *Arabidopsis thaliana* (30, 47). Sequence analysis of this cDNA revealed a high degree of identity (37 to 42%) to published sequences of other eukaryotic glutathione synthetase genes and poor sequence homology to glutathione synthetase genes from prokaryotic sources. The $M_r$ of the polypeptide of 54 to 58 kDa predicted from nucleotide and amino acid sequence

*Table 2.* Properties of glutathione synthetase from higher plants sources (*Nicotiana tabacum, Pisum sativum, Petroselinum crispum, Arabidopsis thaliana*)*

| Biochemical studies | Molecular approaches |
|---|---|
| optimum pH: 8.0 – 9.0 | |
| absolute $Mg^{++}$ requirement | |
| stimulation by $K^+$ | |
| $M_r$: 113 – 120 kDa | |
| homodimer | |
| subunit $M_r$: 68 kDa | $M_r$: 53 – 58 kDa |
| $k_M$ g-glu-cys: 0.02 – 0.07 mM | |
| $k_M$ gly: 0.2 – 1.0 mM | |
| activity in leaves and roots | |
| activity in chloroplasts, proplastids and the cytosol | |
| | mRNA size: 2.5 kb |
| | single gene copy |
| | poor homology to procaryotic sequences |
| | strong homology to eucaryotic sequences |

* data compiled from references 3, 11, 30, 39, 47

analysis was similar to the $M_r$ of glutathione synthetase subunits derived from gel filtration of plant enzyme (Table 2). Apparently, glutathione synthetase is an enzyme highly conserved in evolution of eukaryots. The *Arabidopsis* cDNA isolated functionally complemented the glutathione synthetase deficient mutants of *E. coli* and yeast. Complementation restored the growth rate and significantly enhanced glutathione levels and glutathione synthetase activity (30, 47). Enhanced glutathione levels also restored cadmium tolerance of the yeast mutant complemented with glutathione synthetase cDNA of *Arabidopsis* (47).

## Regulation of glutathione biosynthesis – results achieved by physiological and biochemical studies

At the cellular and subcellular level glutathione synthesis can be controlled (1) by the availability of one of its constituent amino acids, or (2) by the regulation of the activity of the enzymes of glutathione synthesis at the level of expression, translation, or protein functioning.

Cellular cys concentrations are usually low and $k_M$-values of γ-glutamylcysteine synthetase for cys are frequently higher or close to cellular cys concentrations (3). Therefore, *in vivo* glutathione biosynthesis can be determined by the availability of cys for the reaction catalyzed by γ-glutamylcysteine synthetase. This has been observed in yeast, maize, parsley and poplar, where glutathione synthesis is enhanced by exogenous supply of cys or stimulation of cys synthesis (1, 38, 39, 45). At high rates of γ-glutamylcysteine synthesis gly also may become rate limiting for glutathione synthesis. This has been observed with spinach leaves, when fumigation with $H_2S$ had enhanced cys availability and, simultaneously, exposure to darkness had prevented photorespiratory gly production (5, 6). Since gly is thought to be produced primarily in mitochondria and, therefore, has to pass the cytosol on its way to the chloroplasts, γ-glutamylcysteine accumulation as a consequence of a shortage of gly may be of particular significance in the chloroplasts.

In addition to a control by substrate availability, the activity of the enzymes of glutathione synthesis seems also to be regulated, apparently at the level of protein functioning. Similar to the mammalian enzyme (37), plant γ-glutamylcysteine synthetase is inhibited *in vitro* non-allosterically by GSH in concentrations frequently found in cell homogenates (3, 12, 20, 39). Inhibition of γ-glutamylcysteine synthetase by GSH may be overcome in the presence of high glu concentrations, since it was found to be competitive with respect to glu (39). These observations from *in vitro* studies indicate a physiologically relevant feedback mechanism as additional means to control glutathione synthesis. In order to operate also *in vivo*, at least two pre-requisites have to be met by this feedback mechanism. First, inhibition of γ-glutamylcysteine synthetase activity by GSH has to be achieved at the GSH and glu concentrations which prevail at the subcellular location of the enzyme. Second, γ-glutamylcysteine availability is the rate-limiting factor in glutathione biosynthesis. Despite the fact that cys rather than γ-glutamylcysteine availability is rate-limiting for glutathione synthesis in some species (see above), several pieces of evidence suggest that both pre-requisites can be met in plant tissues and feedback inhibition may operate *in vivo*. Cellular γ-glutamylcysteine levels in plants are low indicating that the reaction catalyzed by γ-glutamylcysteine synthetase limits glutathione synthesis (14, 28, 37, 39). When the demand for glutathione is enhanced by cadmium-mediated stimulation of phytochelatin synthesis, cellular glutathione levels are reduced and, at the same time, the rate of glutathione synthesis is increased (3, 39). From these observations it may be assumed that intensive synthesis of phytochelatins can release feedback inhibition of γ-glutamylcysteine synthetase by GSH by reducing the concentration of GSH, and thereby enhances the rate of glutathi-

one synthesis. This interpretation supports the idea that glutathione levels are under the control of GSH-mediated feedback inhibition of γ-glutamylcysteine synthetase. However, improved cys availability or/and enhanced γ-glutamylcysteine synthetase activity seem to contribute to the increased rate of glutathione synthesis upon exposure to cadmium (38, 39). Thus, an unequivocal proof that GSH-mediated feedback inhibition of γ-glutamylcysteine synthetase operates *in vivo* has so far not been achieved.

It is presently unknown as to whether cellular and subcellular glutathione levels are also regulated by expression and/or translation of one of the enzymes of glutathione synthesis. Still, additional mechanisms of control to feedback inhibition by GSH have to be assumed, since cellular glutathione levels are frequently found to be considerably higher than $k_I$-values of GSH for γ-glutamylcysteine synthetase and can be enhanced in a stressful environment (27, 32, 43). The tools of plant molecular biology provide a possibility to address this question. As a first attempt to obtain more clear cut information about the regulation of the pathway of glutathione biosynthesis, poplar plants have recently been transformed with the bacterial gene for γ-glutamylcysteine synthetase, or the bacterial gene for glutathione synthetase (9, 26, 36, 45). Both genes were targetted to the cytosol. Leaves of the transformed poplar plants contained 24- to 80-fold higher γ-glutamylcysteine synthetase, or 15- to 60-fold higher glutathione synthetase activity, respectively, when measured in cell free extracts. The consequences of the overexpression of the enzymes of glutathione biosynthesis for glutathione metabolism are discussed below.

## Metabolic consequences of overexpression of the enzymes of glutathione biosynthesis

### *Levels of glutathione and its metabolic precursors*

Overexpression of γ-glutamylcysteine synthetase in the cytosol caused a more than 3-fold increase in the glutathione level of poplar leaves as compared to untransformed plants (26). Whether the additional glutathione is located in the cytosol, or at least partially in the chloroplast, is at present unknown. Irrespective of the level of γ-glutamylcysteine synthetase activity the oxidized form of glutathione (GSSG) contributed 4 – 5% to total glutathione. Apparently, the GSSG level in the leaves of transformed plants increased proportionally to the total glutathione content. Since foliar glutathione reductase activity was similar in transformed and wild-type plants, the activity of this enzyme in wild-type plants seems to be higher than required to maintain the glutathione pool in its reduced state (26). Foliar γ-glutamylcysteine levels in poplar plants overexpressing γ-glutamylcysteine synthetase were more than 10-fold higher than in wild-type controls. Since glutathione levels were only 3-fold enhanced, it appears that the reaction catalyzed by glutathione synthetase is rate-limiting for glutathione biosynthesis in the transformants. Enhanced contents of glutathione and γ-glutamylcysteine require enhanced availability of cys. Nevertheless, foliar cys contents were similar in transformed and wild-type plants (26). Apparently, the cys level in the leaves of poplar plants is strictly controlled and maintained at a low level, even if the cys requirement is enhanced considerably.

Leaves of poplar plants overexpressing glutathione synthetase 15- to 60-fold in the cytosol contained glutathione levels similar to the wild-type. Cysteine and γ-glutamylcysteine levels were maintained at 3 to 7% and 1 to 2% of the glutathione level, respectively, in both wild-type and transgenic plants (45). From these results it can be concluded that the reaction catalyzed by γ-glutamylcysteine synthetase, but not the reaction catalyzed by glutathione synthetase, limits glutathione biosynthesis in poplar leaves. In

order to obtain information on the nature of this limitation, leaf discs of wild-type and transgenic poplar plants were fed metabolic precursors of glutathione biosynthesis.

*Results of feeding experiments with metabolic precursors*

When leaf discs of wild-type or transformed poplar plants were incubated with water, glutathione levels continue to increase with time of incubation (26). This increase may be a consequence of interrupted long-distance transport of glutathione. Feeding of cys, or cys plus glu, but not feeding of glu, or gly enhanced glutathione accumulation in leaf discs of wild type plants, but a maximum level of 1 to 1.2 μmol $g^{-1}$ fresh weight was maintained despite the accumulation of high amounts of cys inside the tissue (26, 45). Similar results were obtained with leaf discs of transgenic poplar plants overexpressing glutathione synthetase (45). Apparently, availability of cys limits glutathione biosynthesis in the leaves of wild-type poplar, but an additional regulatory mechanism prevents glutathione accumulation above 1.2 μmol $g^{-1}$ fresh weight. This regulatory mechanism was overcome, when γ-glutamylcysteine or γ-glutamylcysteine plus gly were fed to the leaf discs. Under these conditions a continuous increase in glutathione contents was observed. The rate of increase of glutathione was ca. 2-fold higher in leaf discs of transgenic poplar plants overexpressing glutathione synthetase as compared to the wild-type. These results show that glutathione synthesis is regulated at the level of γ-glutamylcysteine synthesis, provided sufficient cys is available. This regulation may be achieved either by feedback regulation of γ-glutamylcysteine synthetase activity as suggested from physiological and biochemical studies (see above), or by the amount of γ-glutamylcysteine synthetase enzyme.

The observation of 3-fold enhanced glutathione levels in leaves of poplar plants overexpressing γ-glutamylcysteine synthetase indicates that an enhanced amount of the enzyme can overcome cys limitation of glutathione biosynthesis. Since feeding of glu did not further enhance foliar glutathione accumulation as compared with exposure to water, but feeding of cys did, feedback regulation by GSH – competitive to glu – of γ-glutamylcysteine synthetase does not seem to control glutathione biosynthesis in these transgenic poplar plants. It may therefore be concluded that in the presence of sufficient amounts of cys the amount of γ-glutamylcysteine synthetase enzyme controls glutathione synthesis. In leaf discs of poplar plants overexpressing this enzyme considerable amounts of γ-glutamylcysteine accumulated in the leaves. This accumulation was further increased when cys was fed. Apparently, high γ-glutamylcysteine synthesis in the transformants has led to a limitation of glutathione synthesis by glutathione synthetase activity (26). If this conclusion is correct, leaves of transgenic plants overexpressing both γ-glutamylcysteine synthetase and glutathione synthetase may accumulate even higher amounts of glutathione than leaves that overexpress γ-glutamylcysteine synthetase, if sufficient amounts of cys are available.

The 3-fold increase in foliar glutathione contents of poplar plants overexpressing γ-glutamylcysteine synthetase is low compared to the 24- to 80-fold elevated *in vitro* γ-glutamylcysteine synthetase activity (26). Similarly, the 2-fold enhanced rate of increase of glutathione accumulation upon feeding of γ-glutamylcysteine to the leaves of poplar plants overexpressing glutathione synthetase is low compared to the 15- to 60-fold elevated *in vitro* glutathione synthetase activity (45). Apparently, most of bacterial γ-glutamylcysteine synthetase or glutathione synthetase activity in the leaves of transformed poplar plants is down-regulated. This down-regulation cannot be attributed to gene silencing at the transcriptional or post-transcriptional level that has frequently been observed in transgenic plants (19), but to inactivation at the level of protein functioning. The nature of this inactivation is presently unknown.

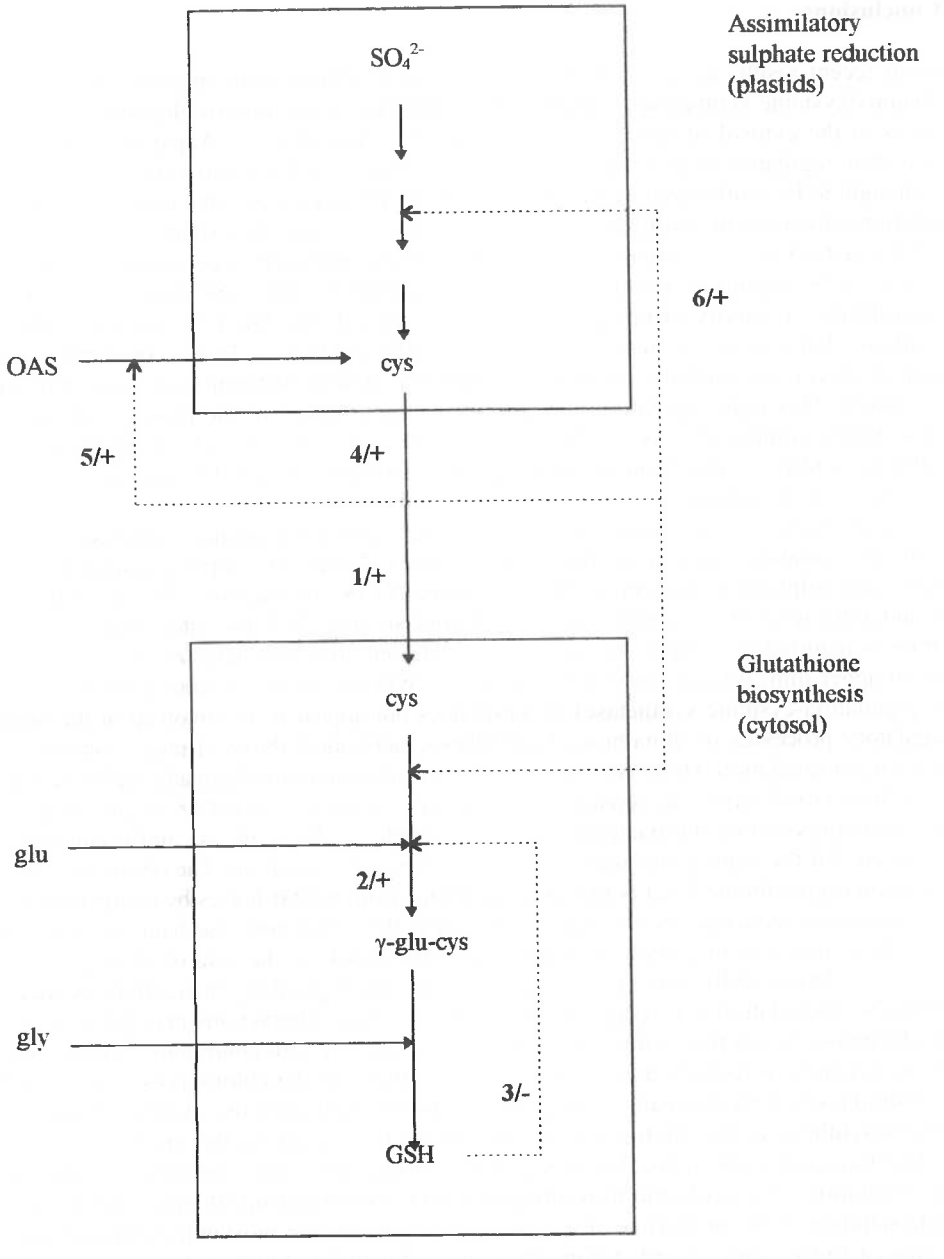


*Fig. 2.* Regulation of glutathione biosynthesis in the cytosol. Broken lines indicate the following regulatory interactions with + for stimulating and – for inhibiting interactions: 1/+, availability of cys; 2/+, γ-glutamylcysteine synthetase activity; 3/-, feedback inhibition of γ-glutamylcysteine synthetase by GSH; 4/+, cys export from the chloroplast; 5/+, interaction of γ-glutamylcysteine synthetase activity with synthesis of OAS; 6/+, interaction of γ-glutamylcysteine synthetase activity with sulphate reduction.

## Conclusions

From recent results achieved with transgenic poplar plants overexpressing bacterial γ-glutamylcysteine synthetase or glutathione synthetase, regulation of glutathione biosynthesis in the cytosol of leaves is a multistep procedure (Fig. 2). Apparently, the most important regulatory step is the availability of cys for glutathione biosynthesis. Since cys is thought to be synthesized in the chloroplasts (4), this regulatory step may be one of the reactions involved in assimilatory sulphate reduction or/and the export of cys from the chloroplasts (Fig. 2). Limitation of glutathione biosynthesis by cys availability can be overcome by regulatory events controlling γ-glutamylcysteine synthetase activity. Elevated levels of activity of this enzyme improve not only the rate of γ-glutamylcysteine synthesis, but also cys availability without affecting cellular cys levels. Apparently, cellular cys levels are carefully controlled irrespective of large differences in fluxes through cys pools. This tight regulation appears to be circumvented in the presence of excess atmospheric sulphur (7). As to whether the actual cys level at the site of enhanced glutathione synthesis also remains unaffected at different fluxes through the cys pool remains to be elucidated.

The interaction of regulatory events controlling γ-glutamylcysteine synthetase activity with cys availability may be located at the level of chloroplastic sulphate assimilation, in particular sulphate reduction or *O*-acetylserine (OAS) production for cys synthesis, or/and at the level of cys export from the chloroplasts (Fig. 2). Thus, inter-organelle regulation is required to achieve this control. In addition, other mechanisms of control may set an upper limit for cellular glutathione levels. Whereas *in vivo* feedback inhibition (as of γ-glutamyl-cysteine synthetase) by GSH does not appear to be involved in the other regulatory processes of glutathione biosynthesis mentioned above, it may contribute to this type of regulation. However, this overall view of regulation of glutathione biosynthesis in the cytosol has to be considered preliminary, since it is based on results achieved by overexpression of the bacterial genes of glutathione biosynthesis and it cannot be excluded that the plant genes undergo different types of regulation. The observation that the cellular glutathione level is increased in tobacco and poplar leaves by overexpression of glutathione reductase (8, 9) supports the view that regulatory mechanisms different from those involved in glutathione biosynthesis participate in the control of the glutathione level. These additional mechanisms may include regulatory interactions between synthesis, degradation and reduction of glutathione. Such interactions may put a ceiling on glutathione levels that is different under normal and stressful conditions. Further studies are required to test whether glutathione biosynthesis in the chloroplasts is controlled by a similar set of mechanisms. Transgenic plants overexpressing the enzymes of glutathione biosynthesis in the chloroplasts will be a useful tool to address this question.

Techniques of plant molecular biology have considerably improved our knowledge on the regulation of glutathione biosynthesis within a short period of time. The present understanding of the properties of the enzymes of glutathione biosynthesis has not been enhanced to the same extent. Information on γ-glutamylcysteine synthetase is largely contradictary to results obtained by biochemical studies. Work on glutathione synthetase has just been started. Progress in this area of research will be an important pre-requisite to unravel further details of the regulation of glutathione biosynthesis and to test whether overexpression of the plant genes of this metabolic pathway leads to the same results as overexpression of the bacterial genes.

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# GENETIC ENGINEERING OF HIGH METHIONINE PROTEINS IN GRAIN LEGUMES

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## Abstract

Progress that conventional plant breeding made in developing nutritionally improved grain legumes has been modest during the past 20 years. After the advent of recombinant DNA techniques and plant transformation two main strategies were followed to engineer high Met grain legumes. 1) Additional Met codons were inserted into isolated storage globulin genes which afterwards were transferred into the plant to be engineered. Although several of these attempts failed, recently the stable formation of engineered 12S and 7S globulins was achieved in transgenic tobacco. Nevertheless, the predictable increases in the Met level of grain legumes is too low to improve their nutritional quality. 2) Foreign genes for Met-rich proteins were transferred into grain legumes like soybean and narbon bean. Transgenic plants were raised which form large amounts of the foreign protein in their seeds. Large increases in seed Met were achieved by this strategy thus demonstrating that the breeding problem can be solved. The new Met sink interacts in a specific way with the plant's sulphur metabolism.

## 1. Introduction

Methionine (Met) and lysine (Lys) are the primary limiting essential amino acids in grain legumes and cereals, respectively. The low level of Met and Lys in seed protein limits their nutritional quality. The Biological Value of seed proteins is 55 to 75% of that of animal references like milk or hen egg protein, depending on the particular legume species. The unbalanced amino acid composition of plant seed proteins limits their efficiency for fattening monogastric animals and poultry and it also contributes to environmental pollution since it entails an inefficient conversion of seed protein nitrogen to animal protein nitrogen that results in a high level of nitrogen excretion. Vegetarians may also be severely affected by the unbalanced amino acid composition of plant seed proteins. In many third world countries, like India and Egypt, the physical and mental development of children up to four years of age can be irreversibly retarded by the deficiency of essential amino acids in their diet.

Plant seeds have been the major source of protein in the human diet since the agricultural revolution. They are consumed either directly, or indirectly after transformation to other proteins by fermentation or feeding to animals. Therefore it is not surprising that improving the nutritional quality of plant seed proteins remains a goal of many legume and cereal grain plant breeding programmes. Progress in developing high Met and high Lys varieties of grain legumes and cereals, respectively, has been relatively modest. Approximately ten years ago, after the advent of genetic engineering and plant transfor-

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mation, several laboratories set out to employ the new techniques to improve the nutritional quality of plant seed proteins. The progress that has been made in engineering high Met legume seeds will be reported in this article.

No similar problems exist with leaf proteins. Their total amino acid composition is balanced like that of animal reference proteins (32) and does not need to be improved from the nutritional point of view.

## 2. Altering the number of methionine residues in 7S and 12S globulins

Globulins according to Osborne (29) represent the major storage proteins in legume grains. Among legume species, the ratio of vicilin-like 7S to legumin-like 12S storage globulins varies. In faba bean (*Vicia faba* L.) 80% of the storage protein is 12S globulin whereas in garden bean (*Phaseolus vulgaris* L.) nearly all storage globulin is 7S globulin. For simplicity 7S and 12S globulins will hereafter be referred to as vicilins and legumins, respectively. Both types of globulins have few sulphur-containing amino acids. Since these storage proteins account for approximately 70 to 80% of the seed protein, they are responsible for the low Met content of legume grains and thus for their limited Biological Value when compared with milk or hen egg protein.

The vicilin holoprotein is a trimer composed of 2 differently sized subunits. The legumin holoprotein is a hexamer composed of similarly-sized subunits (Fig. 1). The subunits of both types of globulins are polymorphic and are encoded by multigene families. The majority of the polymorphic vicilin subunits are free of sulphur-containing amino acids, for example the major 50 kDa vicilin of faba bean. However, there are also some which contain a few SH-amino acid residues, like the subunits of the 50 kDa vicilin of garden bean, called phaseolin. In general, the vicilins are regarded as Met-free. In contrast, legumin subunits always contain at least 4 cysteine (Cys) residues that are involved in intra- and interchain disulphide bridge formation. The inter-chain SS-bridge links the α- and β-polypeptide which form a single legumin subunit (Fig. 1). Legumin subunits may also contain Met. In the faba bean and the pea, two groups of legumin subunits exist. Group A subunits contain Met whereas group B subunits usually do not. In soybean both subunit groups contain Met residues but the group A subunits contain more than group B subunits.

Since storage globulin genes were among the first plant genes to be sequenced (16, 41), several laboratories, including ours, have attempted to increase the number of Met codons in the globulin subunits in order to improve the nutritional quality of legume grains.

### *2.1 Engineering high methionine 12S globulins*

Faba bean legumin contains approximately equal quantities of Met-containing and Met-free subunits (20). Therefore, we attempted to introduce Met residues in the Met-free group B subunits. Both the α- and the β-chain of each subunit have highly variable amino acid sequence regions at the C-terminus which we thought could be altered without affecting the processing and storage functions of the proteins. A frame shift mutation was introduced into the intron-free protein-coding region of a legumin B4 (*LeB4*) gene, 37 codons upstream from the stop codon that terminates β-chain translation. The frame shift generated two Met codons and a premature stop codon which shortened the β-chain C-terminus by 21 amino acid residues. The new stop codon was then converted to a Lys codon by site-specific *in vitro*-mutagenesis. This extended the reading frame (as compared to the original β-chain) by 16 additional nucleotide triplets that included two additional Met codons and a new stop codon. The derived polypeptide sequence of the modified C-termi-

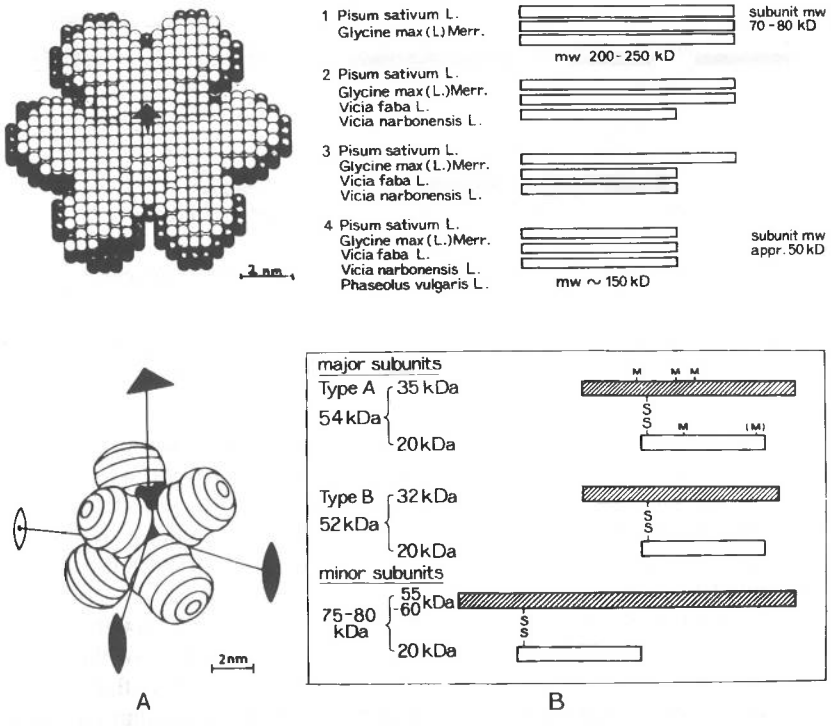


*Fig. 1.* Globulin holoproteins and their subunits (schematically). Size and shape were determined by SAXS (small angle X-ray scattering) in solution (33). (A) 7S globulins (vicilins). Cloverleaf-like model of trimeric phaseolin from *Phaseolus vulgaris* L. Its shape corresponds to an oblate ellipsoid of revolution with axis ratios of 12.5 × 12.5 × 3.75 nm. Different numbers of the two different-sized subunits assemble without disulphide linkages into trimeric holoproteins (25). – (B) 12 S globulins (legumins). Model of the hexameric helianthenin from *Helianthus annuus* L. Each subunit is composed of two disulphide-linked polypeptide chains (α- and β-chains). Six of these similar-sized subunits assemble into the hexameric holoprotein. Its shape corresponds to an oblate ellipsoid of revolution with axis ratios of 12.6 × 12.8 × 8.8 nm (for *Vicia faba* legumin). Two classes of major subunits exist in *V. faba* L.: Met-containing type A- and Met-free type B-subunits. A group of minor subunits is characterized by larger α-chains (25).

nus of the legumin B4 β-chain was 52 amino acids long and contained 4 Met residues. The hydrophobicity profile was similar to the original protein although the total hydrophobicity was slightly greater than in the unmodified legumin β-chain C-terminus (35).

The *LeB4* gene contains three exons and two introns. All the above described changes were in exon 3. This exon corresponds to the 3'-terminal exon of the homologous gene *Gy2* of soybean (*Glycine max* (L.) Merr.) which specifies the G2 (A2B1a) subunit of glycinin, the 12S storage protein of soybean. This subunit contains 7 Met residues, only one of which is located in the C-terminal part of the B1a (β)-chain encoded by the 3'-terminal exon. To construct a *Gy2-LeB4* hybrid gene we substituted the 3'-terminal exon of the soybean *Gy2* gene by the modified 3'-terminal exon of the *LeB4* gene (Fig. 2). The product, called glycigumin gene, coded for a subunit that had 10 Met residues (35).

Both the modified *LeB4* gene with 4 Met codons and the glycigumin gene with 10 Met codons remained under the control of their own promoters and were transferred into tobacco using the *Agrobacterium*/Ti-plasmid system. Transcripts of both genes could be detected in transformants containing the modified genes but the corresponding proteins

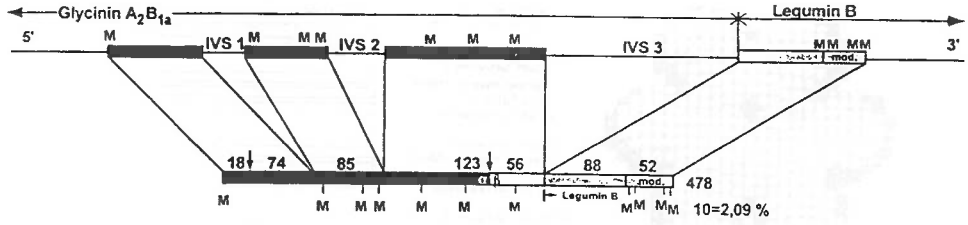


*Fig. 2.* Engineering of Met codons into the 3'-terminal exon of *V. faba* legumin *LeB4* gene (upper line right) and construction of the glycinin *Gy2-LeB4* hybrid gene "glycigumin". The 3 introns are derived from the *Gy2* gene. The corresponding pre-proglycigumin polypeptide with its 11 Met residues is indicated in the lower line. Since the N-terminal signal peptide (18) with its initiator Met is lost by cotranslational detachment the mature subunit contains 10 Met residues corresponding to 2.09% of the amino acid residues.

were never detected. Since the same constructs produced polypeptides in transgenic yeast as well as in a coupled *in vitro*-transcription/translation system, we concluded that the gene constructs were functional and the proteins could not be detected because they were rapidly degraded. We were later able to show that the mutated C-terminus of the legumin β-chain interfered with correct subunit folding and opened the polypetides to premature degradation (36).

In Utsumi's group in Kyoto regions in 12S globulin subunits were sought in which the introduction of Met residues should not interfere with correct folding of the polypeptide and its assembly into the holoprotein. Clusters of 3 to 4 Met codons flanked by single amino acid residues have been inserted into different sites of the glycinin *Gy1* gene (Fig. 3). Similar to all other 12S globulin genes it encodes the common precursor of the glycinin subunit G1 with α- and β-chains still in peptide linkage. These proglycinin polypeptides can only assemble into trimers but not into hexamers like mature subunits (12). The constructs were transferred into *E. coli* where it was possible to demonstrate that the expressed proglycinin polypeptides correctly assembled into trimers like the corresponding wild-type glycinin subunit precursors. Two sites were found in the glycinin subunit that tolerate such modifications: one the C-terminal variable region of the α-chain, and a second one very close to the C-terminus of the β-chain. In transgenic tobacco seeds (42) and potato tubers (45) the analogous glycinin subunit mutations turned out to be stable and no difference was found between the expression levels of wild-type and mutated glycinin (17).

### *2.2 Genetic engineering of high methionine7S globulin*

Comparison of sequenced vicilin-specific DNAs from several plant species indicated that Met codons are present in 5 different positions scattered throughout different genes. As mentioned above, the major faba bean vicilin subunit is free of SH-amino acid residues. Therefore, we projected the known 5 Met codon sites into the homologous positions of the faba bean pVic 91 cDNA (7) which corresponds to vicilin gene *VfVic1* (47). In addition, 3 positions were selected where, with a high probability, a conservative amino acid exchange should not affect the functionality of the vicilin subunit. Eight Met codons were generated by site-specific *in vitro*-mutagenesis (Fig. 4) at the selected sites. The construct was put under the control of the seed-specific USP-promoter from *V. faba* (5) and transformed into tobacco using the *Agrobacterium* system (36).

In this experiment it was possible to detect the altered protein in transgenic tobacco seeds. It could be distinguished from wild-type vicilin by its cyanogen bromide (CNBr) cleavage pattern since it cleaves specifically at Met residues. The wild-type protein is not

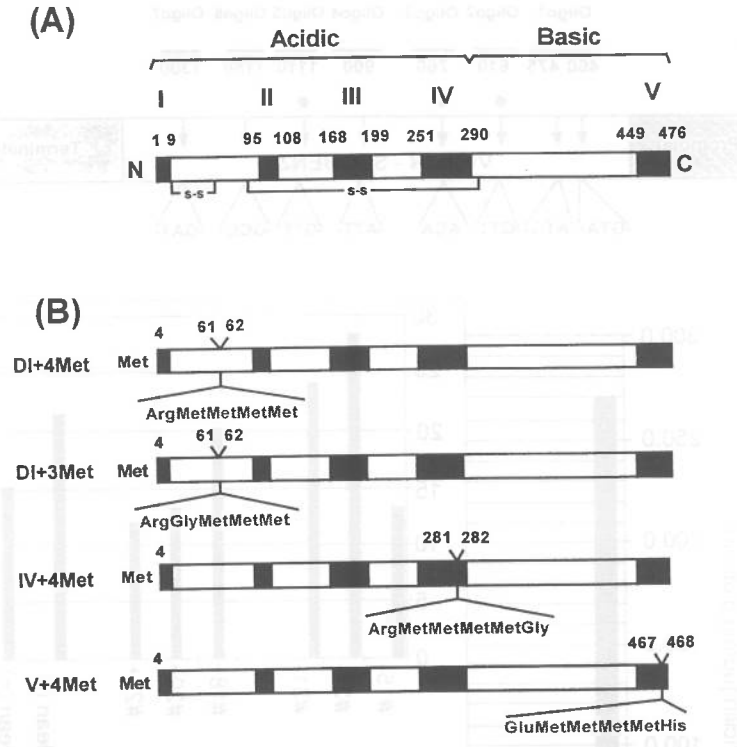


*Fig. 3* Engineering of Met codons into the soybean subunit G1 (corresponding to gene *Gy1*) gene. (A) The structure of the wild-type precursor polypeptide of G1 with its conserved (white bars) and variable (black bars I – V) regions as well as the position numbers of border amino acid residues of the variable regions are indicated. The G1 subunit precursor consists of a signal peptide with 19 amino acid residues, the α- and β-chain of the subunit in peptide linkage. Sites of Cys codons are indicated where disulphide bridges are formed in the subunit. – (B) A selection of constructs with clusters of 3 or 4 Met codons inserted at different sites in the conserved (DI+4Met and DI+3Met) or variable regions (IV+4Met and V+4Met) of the polypeptide. Trimer formation took place with constructs IV+4Met and V+4Met where insertions were located in the highly variable C-terminal region of the α-chain and very close to the C-terminus of the β-chain, respectively (46).

cleaved. The modified vicilin chain produced the expected cleavage products. Faba bean vicilins could be specifically quantified by ELISA techniques in extracts from in the transgenic tobacco seeds. No statistically significant difference was found between levels of the wild-type and mutated vicilin polypeptides in seeds of tobacco transformants harbouring the wild-type (control) and mutated gene, respectively (Fig 4). These experiments confirmed that it is possible to introduce additional Met residues into globulin polypeptides without affecting the protein's functionality.

### *2.3 Perspectives*

The genetic engineering of high Met-globulin polypeptides has several drawbacks besides the fact that most alterations affect either the protein's ability to be properly transported, processed, and deposited in the storage tissue cells of developing seeds or its ability to be reactivated upon germination.

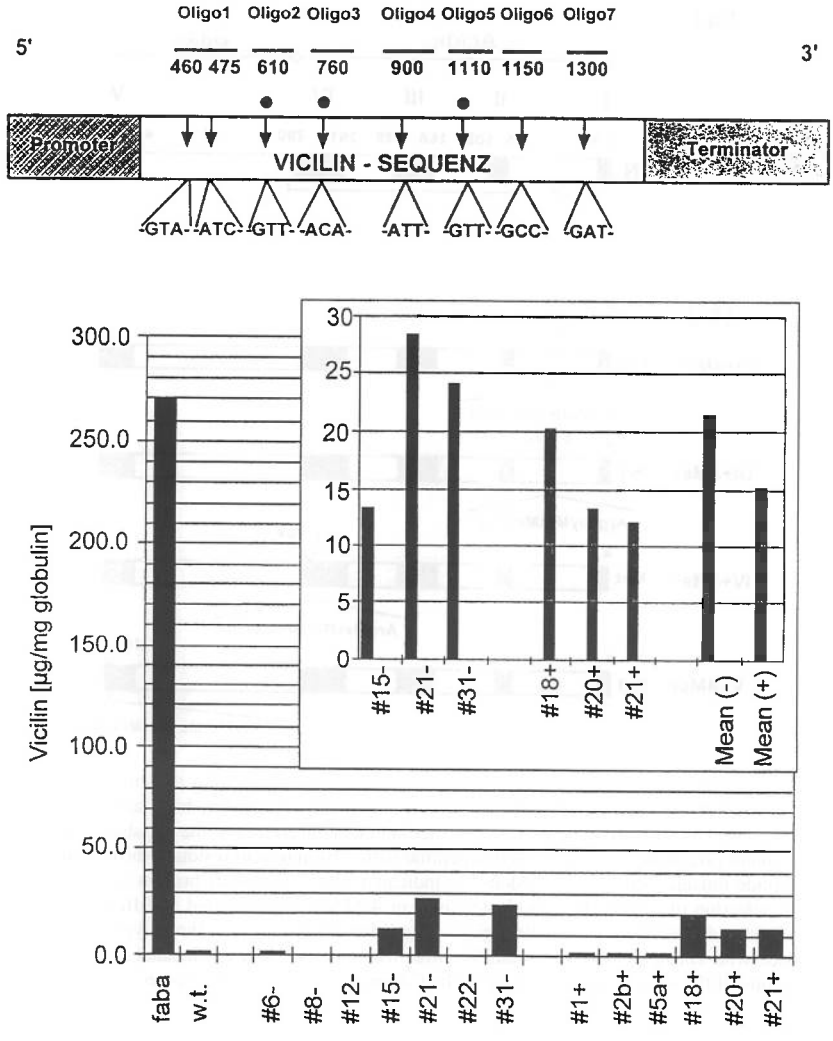


*Fig. 4.* Engineering of Met codons into the major vicilin gene of *V. faba* which originally is entirely free of Cys and Met codons. (A) Positions in the pVi91 cDNA (intron-free *VfVic1* gene) where codons have been mutated into Met codons. Dots above arrows indicate the 3 conservative amino acid exchanges that were generated in addition to the naturally occuring Met positions in homologous 7S globulin genes of other legumes. – (B) Analysis of seeds from different transgenic tobacco lines harbouring either the wild-type (-) or the mutated (+) vicilin gene. For comparison the vicilin quantity of faba bean is given. The insert documents the ELISA-based quantitative comparison of three transformants each with the wild-type and with the mutated gene. No significant difference could be determined.

a) Multigene families encode the polymorphic subunits of globulins. Since only one of the gene families' members is altered, it will be difficult to significantly increase the Met content of the seeds.
b) Although it may appear that we have introduced a large number of Met residues in the glycigumin and modified vicilin subunit constructs, this number is still much to small to raise the seed's total Met content by the factor of 2 that would be required

for economic use. The successful introduction of greater numbers of Met residues has so far not been reported by other authors (17).

c) Replacement of the wild-type copies with high Met copies *via* homologous gene exchange could theoretically improve the situation. However, in plants it is not yet possible to use homologous recombination to either knock out or exchange genes.

Taken together, the results so far achieved by engineering high-Met subunits from naturally occurring globulins indicate that it will not be possible to achieve significant increases in the Met content of the seed protein *via* this strategy in the near future. An alternative strategy would be to engineer a synthetic high Met seed protein using our knowledge of the structural requirements of seed storage proteins. This strategy is still in its infancy since only transformation experiments with genes that have been isolated from plants have so far been published.

## 3. Transfer of genes for methionine-rich foreign proteins

Currently the most promising strategy to improve Met content is to introduce genes that have been isolated from foreign plants and that specify proteins with extremely high Met levels. The favored subjects for such experiments are the genes encoding 2S albumins from either Brazil nut, *Bertholletia excelsa* H.B.K. (1) or sunflower, *Helianthus annuus* L. (21, 22). The feasibility of this strategy was initially shown by using the Brazil nut 2S albumin gene (BNG) to transform tobacco and rape (2, 3). In our laboratory a 2S-albumin gene was chemically synthesized (38) according to the published cDNA sequence (1) and used to transform the narbon bean, *Vicia narbonensis* L. The experiments have been accomplished using an *Agrobacterium tumefaciens*-mediated transformation system that had been developed for this Mediterranean grain legume (30, 31). The narbon bean is closely related to the faba bean (*V. faba* L.) for which an effective transformation/regeneration system has not yet been established. Workers at Pioneer Hi-Bred Inc., USA, have since successfully used the Brazil nut albumin gene to transform an economically important grain legume, soybean (44).

### *3.1 Formation of the Met-rich Brazil nut 2S albumin (BNA) in transgenic narbon bean*

BNA contains 18% methionine and 8% cysteine residues which makes it one of the most sulphur-rich proteins known. It is a genuine seed storage protein and is stored in protein bodies located within the hypocotyl of Brazil nuts. The mature protein is composed of two subunits: a large (9 kDa) and a small (3 kDa) chain that are joined by disulphide bridges. Both polypeptides are derived by the processing of a single larger common precursor which is formed on the rough endoplasmic reticulum (RER) in the storage tissue cells. The process of its stepwise molecular maturation is schematically presented in Fig. 5.

#### 3.1.1 Gene construct and transformation

Our synthetic BNA gene contains 441 nucleotides and encodes the complete 146 amino acids long BNA pre-propolypeptide (38). This gene was put under the control of the *LeB4* gene promoter from *V. faba* (4, 6) which confers developmentally regulated tissue specific expression in seeds. After transfer into the binary Ti-plasmid vector pGA472, the BNG construct was transferred into narbon bean epicotyl explants using the *Agrobacterium* system. After callus formation plants were regenerated *via* somatic embryogenesis. Cloning of transformants could be done *via* secondary somatic embryos. Since rooting of the plants was difficult, young shoots were grafted on seedling axes (for details see 31).

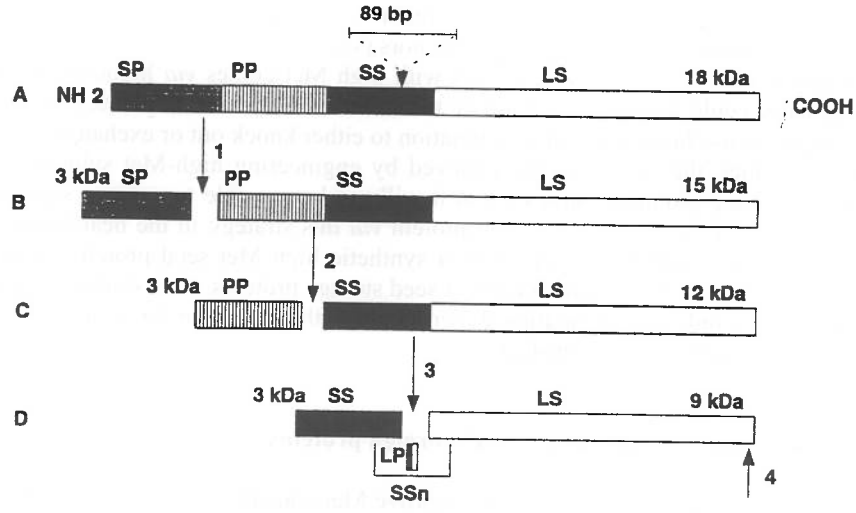


*Fig. 5.* Generation of mature BNA by precursor polypeptide processing. The primary translation product (A) is an 18 kDa pre-propolypeptide. It is processed by a series of limited proteolytic steps. The first step cotranslationally removes the 3 kDa N-terminal signal peptide (SP) after polypeptide insertion into the endoplasmic reticulum (B). The 3 kDa propeptide (PP) is split off in the next step (C) which like the subsequent ones presumably takes place in the storage vacuole. There, a linking peptide is removed (D) to yield the mature 9 (LS) and 3 kDa polypeptides (SS) which remain linked by disulphide bridges. Finally, a tetrapeptide is detached from the C-terminus of the 9 kDa chain. This peptide is essential for the vacuolar targeting of BNA (37).

### 3.1.2 Gene copy numbers and inheritance

Of 70 independent transgenic narbon bean $R_0$ plants, 50 expressed BNA. Segregation and Southern analysis have been performed with 18 of these plants. Sixteen of these transformants formed BNA in their seeds. Inheritance of the foreign gene, copy number and stability of gene expression have so far been analyzed in the offspring of 13 $R_0$ plants. The segregation analysis was consistent with Mendelian inheritance of single gene inserts. Instability of the gene expression was preferentially observed in the offspring of transformants with more than one gene insertion. Homozygous lines with single inserts were obtained from 3 transformants. A tandem insert inherited as a single locus was found in an additional line. Probes spanning both the left and right T-DNA borders were employed in Southern hybridization which indicated the integration of the BNG into the narbon bean genome (31, 39).

### 3.1.3 Gene expression and BNA accumulation level

Gene expression was analyzed by SDS polyacrylamide gel electrophoresis. The Coomassie stained BNA band was quantified using computer based image analysis. Immunogold-labeling of thin sections in the electron microscope was used to show the vacuolar localization of BNA (26, 40). Whereas BNA was found in seed extracts by immunoblotting, it could not be detected in extracts from stems, leaves and roots. This result confirmed that the *LeB4* gene promoter also confers seed-specific gene expression in narbon bean. Identical developmental patterns have been observed at the level of mRNA as well as polypeptide formation for BNA and legumin. This was to be expected due to the similarity of

promoters which control the formation of both proteins in the transgenic narbon bean. Antibodies against BNA reacted with 9 and 3 kDa polypeptides in Western blots, thus indicating that in narbon bean the pre-propolypeptide which must have been formed as a primary translation product was processed into the mature protein as in the Brazil nut.

In seeds of the 3 homozygous lines with single gene inserts, BNA amounted to 1 to 3% of the total SDS-soluble protein. Another putative homozygous line contained 6% BNA, which was the highest level of the foreign protein we have observed so far. Unfortunately, the seeds of this line were not viable and the callus culture was lost. In the homozygous line A8 which contains a tandem insert approximately 4.8% of the SDS-soluble seed protein was BNA. This level has been stably inherited through 9 generations. In three investigated cases the homozygous lines produced approximately twice the amount of BNA as do the corresponding heterozygous plants, indicating that the expression level is proportional to gene dosage.

### *3.2 Effects on the methionine level in transgenic narbon bean*

The expected increase of Met in transgenic narbon bean was calculated from the amounts of BNA in the different homozygous lines that had been determined by Commassie staining, and assuming that the amino acid composition of the remaining proteins did not change. Results from amino acid analysis of seed flour agreed with these calculations (Fig. 6). This indicates that the total Met content of endogenous narbon bean proteins remained unaffected by the formation of the foreign protein. In line A8 with the tandem insert and 4.8% BNA in the SDS-extractable protein the total Met content of seed flour was doubled in comparison with the wild-type narbon bean (Fig. 7) whereas the other amino acids remained nearly unchanged. It reached the Met level of soybean which corresponds to 80% of the FAO standard for a nutritionally balanced protein. Therefore we conclude that the nutritional quality of legume seeds can be significantly improved using this strategy.

The percentage increase of the Met levels in our transgenic narbon bean line A8 appears to be very large when it is compared with the increases of 30 to 40% that have been reported from similar transformation experiments with tobacco (2) and rape (3). However, the level of Met in the salt-soluble protein fraction of wild-type *V. narbonensis* seeds is only 0.5% (mol/mol), whereas the corresponding protein fraction of the wild-type winter variety of *Brassica napus* that was used by Altenbach et al. (3) contains 2.64% (mol/mol) Met. The absolute increases in the Met levels of transgenic narbon bean and rape are approximately the same.

The breeding company Pioneer HiBred International (USA) has reported on a soybean line in which 10% of the salt-extractable seed protein was BNA. However, seed flour from this line showed only a thirty per cent increase in the Met content as compared to the wild-type (44). Here it appears that the formation of some endogenous Met-containing proteins is suppressed by the metabolic demands imposed by expressing high levels of BNA. Indeed, they have been able to identify in two dimensional gels several bands that are significantly decreased in intensity in transgenic lines. One of the bands has been identified as a Met-rich protease inhibitor protein.

### *3.3 Effects on other endogenous sulphur-containing compounds in transgenic narbon bean*

Although the total Met content is doubled in the seeds of transgenic narbon bean line A8 with 4.8% BNA in the SDS-extractable protein, their total sulphur content was similar to that of wild-type seeds. This suggests that the increase of the Met level occurred at the cost of other non-protein S-compounds. *V. narbonensis* seeds have been shown to be

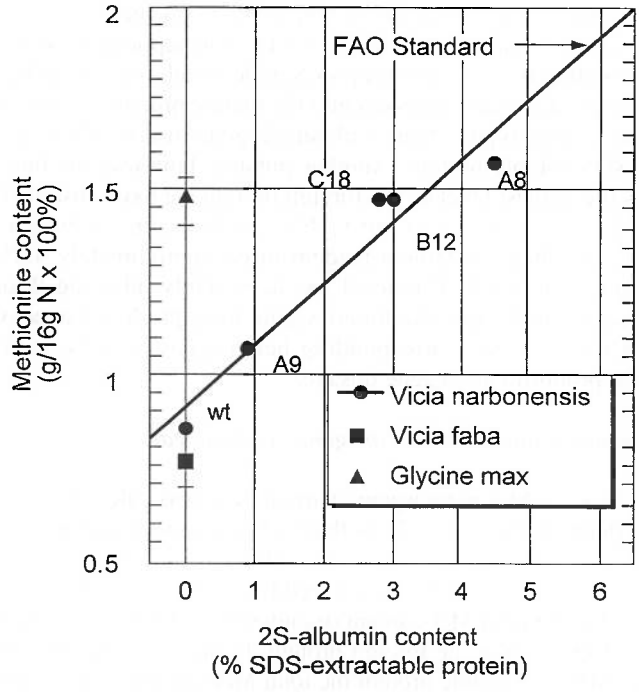


*Fig. 6.* Methionine content of the homozygous wild-type and of 4 different homozygous transgenic lines of narbon bean as a function of the amount of 2S albumin. Whereas lines A9, B12 and C18 harbor single gene inserts line A8 contains a tandem insert which is inherited as a single locus. The Met content of *V. faba* and *Glycine max* as well as the FAO standard for the Met content of a nutritionally balanced protein are indicated for comparison. The increase of Met content in transgenic seeds is directly proportional to the level of 2S albumin formation.

much richer in sulphur (0.28 – 0.37% of dry matter) than the seeds of other grain legumes, like faba bean, pea, soybean or lupin (0.16 – 0.25%). Indeed, Enneking (13) found that narbon beans accumulate the dipeptide γ-glutamyl-S-ethenyl-cysteine (GEC) which forms the basis for their high sulphur content. At least under sulphur limitation seeds of our homozygous transgenic narbon bean lines contained less GEC than wild-type seeds. The decrease in the amount of GEC-bound sulphur seems to compensate the sulphur increase in BNA. These results still have to be confirmed by repetition in subsequent experiments. Independent of whether or not the additional sulphur required for BNA synthesis comes from GEC, the metabolic channelling of sulphur from one compartment to another within the seed might at least in part to provide the additional sulphur required for BNA synthesis.

### *3.4 Endogenous methionine supply and the formation of foreign methionine rich proteins in transgenic legume seeds*

In transgenic soybean as well as narbon bean high levels of the Met-rich BNA are formed at the expense of other endogenous S-containing compounds. The BNA acts as a strong S-sink which in soybean presumably out-competes the formation of at least some of the other Met-containing endogenous proteins. In narbon beans GEC may provide an alternative source of extra sulphur for the high level BNA formation. In both cases the available sulphur may limit the total amount of sulphur-containing compounds that can

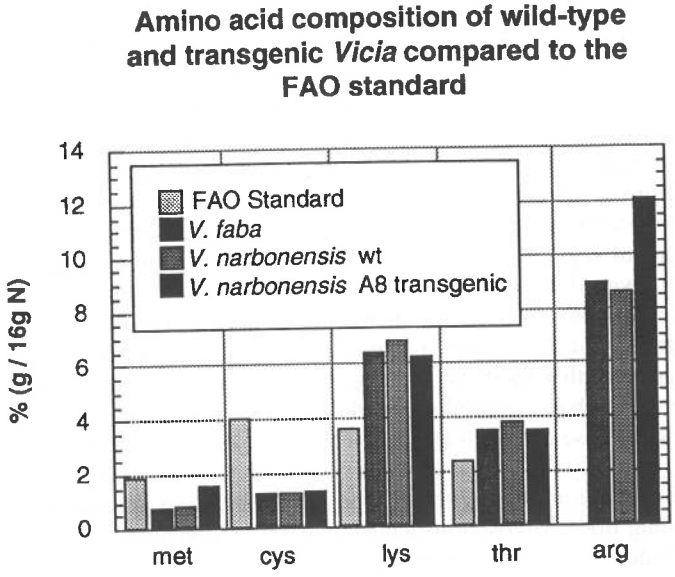


*Fig. 7.* Amino acid composition of wild-type and transgenic *V. narbonensis,* line A8, compared to the FAO standard for a nutritionally balanced protein. Only amino acids synthesized in the aspartate pathway, cysteine and arginine are included in the figure.

be formed in transgenic seeds with high levels of the S-rich BNA. It would be interesting to know whether in cotyledon culture or in the whole plant, an additional S-supply could prevent the reallocation of sulphur among the endogenous S-containing compartments of the seed. This is related to the more general question of how the metabolism of sulphur is regulated in transgenic seeds. This question has to be experimentally answered in the future. However, the published results on the influence of sulphur starvation on the formation of storage globulins in legume seeds have already given us some indications concerning regulatory principles.

### 3.4.1 Hypotheses on the control of gene expression by sulphur-nutrition

BNG was put under the control of the phaseolin promoter to be transferred into soybean, whereas we used the *LeB4* gene promoter-BNG construct for narbon bean transformation. Phaseolin is the vicilin-like 7S globulin which strongly predominates in seeds of garden bean (*Phaseolus vulgaris* L.). Legumin B4 is a major representative of the 12S storage globulins from field bean (*Vicia faba* L.). Unfortunately, neither the S-dependent regulation of the expression of phaseolin genes in garden beans nor of the legumin B4 genes in field beans has so far been investigated. Detailed experiments have been performed on the influence of the level of S-supply on the formation of 7S and 12S globulins and their subunits in developing pea, lupin and soybean.

As mentioned above (section 2), the 7S globulins are much poorer in sulphur than 12S globulins although both only contain small amounts of SH-amino acid residues. Under conditions of S-deficiency, legumin formation was decreased and vicilin formation was increased in pea seeds. Therefore, the globulin concentration remained nearly constant if the degree of S-deficiency did not exceed certain limits (10, 11, 34). Similar results were reported from corresponding experiments with lupins (9) and soybean (15). The 7S glob-

ulin of soybean, which is named β-conglycinin, has trimeric holoproteins (see also Fig. 1) composed of different ratios of a 50 kDa subunit (β-chain of β-conglycinin) and two larger subunits (α- and α'-chains of β-conglycinin). Whereas the formation of α- and α'-chains of β-conglycinin remained nearly unaffected by the reversible influence of S-deficiency, the formation of β-chains was increased under S-deficiency and decreased during recovery from S-deficiency. This has been demonstrated using cotyledon culture in nutrient solutions with different levels of S-supply (43) as well as in intact soybean plants (15). If the corresponding genes were transferred into petunia or *Arabidopsis thaliana* the pattern of regulatory dependence on S-supply was maintained in the trangenic seeds (14, 27). Even if the promoters of the β-conglycinin chains were fused to reporter genes, their expression in transgenic plants was similarly dependent on the degree of S-nutrition regardless of how sulphur was supplied, as sulphate or methionine (18, 19). Therefore, it appears that the S-dependent expression of genes for β-conglycinin chains, especially for the β-chain, is predominantly regulated at the transcriptional level. These results agree with those obtained by Chandler et al. (10, 11) for peas. In seeds of S-deficient plants a relatively high level of vicilin synthesis was observed throughout the entire phase of protein accumulation whereas legumin formation was reduced. The changes in the corresponding mRNA closely reflected the pattern of synthesis of the vicilin and legumin polypeptides. Run-off transcription experiments were employed to compare the levels of vicilin- and legumin-mRNAs available for translation with corresponding transcriptional activities in isolated nuclei (8). In developing seeds of S-deficient plants mRNA levels agreed with transcription levels of corresponding genes. Under conditions of recovery from S-deficiency the transcription of vicilin genes decreased strongly. Although under similar conditions legumin gene transcription approximately doubled the amount of translatable mRNA increased by a much larger factor. This result indicated that under conditions of recovery from S-deficiency post-transcriptional regulation plays an important role besides transcriptional control in the expression of legumin genes. It remains open if under these conditions Met activation by tRNA binding becomes a translation limiting factor. However, no indication of such limitations was found in experiments with S-deficient peas (23). Regulation of the expression of other genes encoding S-rich proteins like proteinase inhibitors of soybean (43) as well as albumins of pea (11) has also been reported to depend on sulphur supply. From experiments on the mechanism of hordein C-gene regulation in developing barley grains it is known that storage protein promoters contain modules which mediate the positive and negative control of transcriptional activity by amino acid levels. The limiting essential amino acid of many cereal grains is lysine, and it appears to play an important role in regulating transcription of prolamin genes in these plants (24). One could speculate that similar control elements could exist for S-containing amino acids in globulin genes of legumes.

### 3.4.2 Control of 2S albumin gene (BNG) expression by phaseolin promoter

Since in transgenic seeds with high levels of BNA the S-supply seems to limit the total amount of S-compounds that can be formed, conditions for gene expression should mimic limited S-deficiency.

Provided the phaseolin promoter reacts similarly to the homologous vicilin promoters of pea and β-conglycinin β-chain promoter of soybean, the expression level of the gene fused behind this promoter should be increased independently of whether it encodes an S-rich or S-poor protein. Similarly, the expression of other genes should be repressed if their promoter activity were down-regulated under conditions of limited S-supply. BNG transcription analysis should be performed with BNA-forming soybean to verify this

hypothesis. Met-dependent regulation of the level of Bowman-Birk proteinase inhibitor mRNA was reported for *in vitro*-cultured soybean cotyledons (43). This agrees with the finding that proteinase inhibitor levels are decreased in transgenic soybean with high levels of BNA accumulation (44).

### 3.4.3 Control of 2S albumine gene (BNG) expression by legumin B promoter

Field bean, the donor of our *LeB4* gene promoter, and peas, which were used in Chandler's and Beach's experiments on the influence of S-nutrition on storage globulin formation, are taxonomically closely related, e.g. legumin A subunits of both legumes exhibit up to 95% sequence similarity (48). Nevertheless, the effects found with our LeB4-BNG construct in narbon bean cannot be explained by a correspondingly simple analogy to the S-dependent regulation of legumin gene expression in pea as suggested for the phaseolin promoter. As mentioned above, under limited S-deficiency legumin gene expression was repressed in pea, whereas we found incorporation of Met into BNA at the expense of GEC formation in narbon bean. Pea and field bean legumin differ in their composition of polymorphic subunits. Met-free legumin B subunits represent a very small percentage and legumin A strongly predominates in pea legumin. In field bean the legumin B subunits amount to approximately 50 percent of the total legumin. If legumin A and B genes were differently controlled by S-deficiency this could not have been observed in Chandler's and Beach's early experiments with pea. In addition, the existence of Met-containing legumin A- and Met-free legumin B-subunits was still unknown in 1984 (11) and 1985 (8). Provided in field bean the control of legumin A and B gene expression by sulphur deficiency corresponds to that of Met-codon-containing legumin A and Met-codon-free vicilin genes, respectively, of pea, then it has to be expected that the *LeB4* gene promoters mediate up-regulation and the legumin A promoters down-regulation under limited S-supply. Provided that this is the case, the channelling of sulphur into BNA formation at the expense of GEC synthesis in transgenic narbon bean could be explained by preferential regulation at the transcriptional level. This is all still pure speculation, but it might be used as a working hypothesis to be verified experimentally.

### 3.4.4 Significance of γ-glutamyl-S-ethenyl-cysteine (GEC) in transgenic narbon bean

Finally, it should be mentioned that nothing is known about anabolism and catabolism of GEC which structurally is related to glutathione (γ-glutamyl-cysteinyl-glycine). Analysis of the enzymes of these pathways and their genes will provide the tools to analyze the control of GEC metabolism by S-nutrition.

GEC was shown to be unpalatable to pigs and this is the principal factor preventing the more widespread use of *Vicia narbonensis* as fodder (13). Therefore, strategies to decrease GEC are sought. If indeed BNA formation decreases the quantity of GEC in transgenic narbon bean, as our initial results suggested, then two nutritional aspects of the grain legume would be improved through the introduction of the BNA gene. Presumably, further increases of the BNA content of transgenic narbon bean could lead concomitantly to even more decreased amounts of GEC thus combining improvement of the nutritional value as well as the palatability of this grain legume. Common interest exists of the Australian Centre for Mediterranean Legumes in Agriculture (CLIMA) with our group to test the practical feasibility of this strategy.

## 4. Conclusions

Most of the economically important grain legumes, like pea, garden bean, or lupin, have low S-contents like soybean and do not contain GEC or similar low molecular weight S-deposits. Therefore, in accordance with the results reported for transgenic soybean it has to be expected that expression of high levels of foreign Met-rich protein in transgenic seeds of these legumes may suppress the formation of endogenous S-containing proteins. Surprisingly little is known about sulphur supply to and about pathways, control and regulation of sulphur metabolism in plant seeds. Consequently, investigating the regulation of S-metabolism now becomes not only an interesting goal in basic research, but also should contribute to solving the problem of engineering high Met grain legumes. Transgenic high Met grain legumes appear to be useful tools in research on the regulation of S-metabolism in legume seeds.

Further use of BNG for the engineering of high Met grain legumes has to be questioned since BNA turned out to be the major allergen of Brazil nut (28). This finding provoked new arguments against employing genetic engineering for food improvement. Of course, all Met-rich proteins that could be candidates for the improvement of the amino acid balance in grain legumes will similarly undergo allergenicity testing as was done with BNA. No allergens should get into engineered plants which are intended for commercial use in food. In addition, the allergenicity problem challenged new research to find non-allergenic Met-rich proteins or to eliminate allergenic domains of BNA by protein engineering. Furthermore, research on possibilities of engineering the ratios between endogenous Met-rich and Met-poor proteins of grain legumes received new impulses. This makes basic studies on promoters of seed protein genes even more interesting. Again basic and applied research, which really cannot be separated, are activating each other.

Commercially even more important is the engineering of high Lys maize, wheat, barley or sorghum but no progress similar to the engineering of high Met grain legumes has so far been reported. At present no commercial grain legume variety with improved amino acid composition is available for the reasons described above. Nevertheless, our model experiments with narbon bean especially have shown that the engineering of high Met grain legumes represents a realistic approach to resolve the problem of unbalanced amino acid composition of legume seed proteins, and it can be taken as an indication that the low Lys problem of maize and several cereals might similarly be resolved by genetic engineering.

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# MODIFYING THE SULPHUR AMINO ACID CONTENT OF PROTEIN IN TRANSGENIC LEGUMES

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## Abstract

Chimeric genes encoding a sunflower seed albumin (SSA) rich in the nutritionally essential amino acids, methionine and cysteine, were transferred to tobacco, lucerne and subterranean clover using *Agrobacterium tumefaciens*. In the vegetative parts of transgenic subterranean clover plants, SSA accumulated to levels estimated to supply rumen-protected sulphur amino acids in quantities similar to those reported to result in increased wool growth in sheep. A gene encoding SSA was transferred to lupin, and accumulation of the protein in transgenic seeds was associated with increases in seed sulphur amino acid content and nutritional value.

## Introduction

Animals are incapable of synthesising ten of the twenty amino acids needed for protein synthesis. These so-called essential amino acids must consequently be obtained from the diet. In order to obtain maximal efficiency of feed protein utilisation, the intake of the essential amino acids must be balanced with respect to each other. For this reason, animal feed formulations usually include protein from diverse sources. Cereal grains, commonly used as the principal energy source in the diet, contain protein poor in lysine (1). Conversely, legume seed protein generally contains an adequate supply of lysine, but is deficient in the sulphur amino acids, methionine and cysteine (2). Deficits in essential amino acids which can consequently occur in formulated diets for animals are often rectified by the addition of pure, crystalline amino acids.

With the advent of gene transfer technology, it is now possible to think of enhancing the levels of essential amino acids in some of the plant material commonly used as animal feed. For example, proteins with unusually high contents of methionine and cysteine have been identified, and their structural genes cloned (3, 4). Genes encoding a methionine- and cysteine-rich Brazil nut seed 2S storage protein have been transferred to canola (5) and narbon bean (6) resulting in seed-specific expression of the introduced genes, and concomitant increases in sulphur amino acid content of seed protein. One concern regarding the use of the Brazil nut protein for nutritional enhancement, particularly of material for human consumption, is the recently demonstrated allergenicity of the protein in some subjects (7).

[3] We dedicate this report to the memory of Dr. Bjorn Eggum who died after a short illness on March 12th 1996

*Sulphur Metabolism in Higher Plants, pp. 87-93*
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We report the transfer of a constitutively-expressed chimeric gene encoding a methionine- and cysteine-rich sunflower seed storage protein to the pasture plant, subterranean clover (*Trifolium subterraneum*) in order to improve the nutritional value of the leaf and stem protein for ruminant animals. In addition, we have transferred a seed-specific gene encoding the sunflower protein to the narrow leaf lupin, (*Lupinus angustifolius*), pea (*Pisum sativum*), and chickpea (*Cicer arietinum*) for enhancement of the sulphur amino acid content of the seed protein, and hence its nutritional value.

## Adding proteins rich in sulphur amino acids to the leaves of pasture legumes

It is especially difficult to accurately formulate the diets of ruminant animals. The protein ingested by ruminants is largely converted to microbial protein during fermentation in the rumen. This conversion process utilises the ingested protein relatively inefficiently, with significant losses of nitrogen and sulphur occurring before the eventual degradation and assimilation of the microbial protein in the lower gastro-intestinal tract of the animal (Fig. 1). A small number of proteins have been shown to be naturally resistant to degradation by the rumen microflora, by virtue of their secondary structures (10). Such proteins, referred to as "bypass protein", travel to the true stomach, or abomasum, of the animal in a relatively intact form. There, they are degraded and their amino acids are absorbed directly into the animal's bloodstream.

Nutritional studies involving feeding of protein that is chemically protected against degradation in the rumen demonstrate that ruminant animal production is often limited by amino acid supply (11, 12). Furthermore, post-ruminal infusion of specific amino acids, has been reported to improve animal production. In particular, post-ruminal infusion of methionine and cysteine in sheep can result in large increases in wool growth rate (Fig 2, and refs 13 and 14).

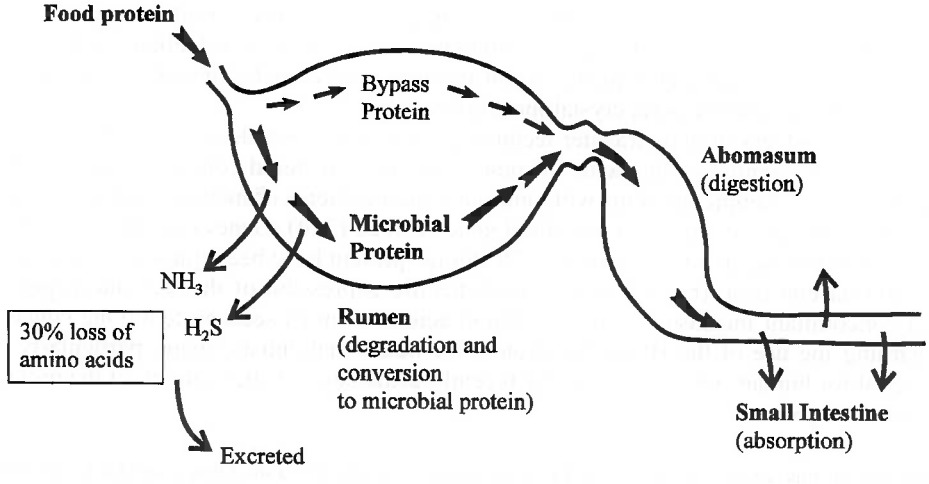


*Fig. 1.* The fate of dietary protein in sheep. In the rumen, ingested protein is degraded to ammonia, approximately 70% of which is converted to microbial protein. The remaining 30% is absorbed across the rumen wall into the bloodstream, and eventually excreted in the form of urea (8). A similar proportion of unutilised sulphur from degraded protein is lost from the rumen as hydrogen sulphide (9).

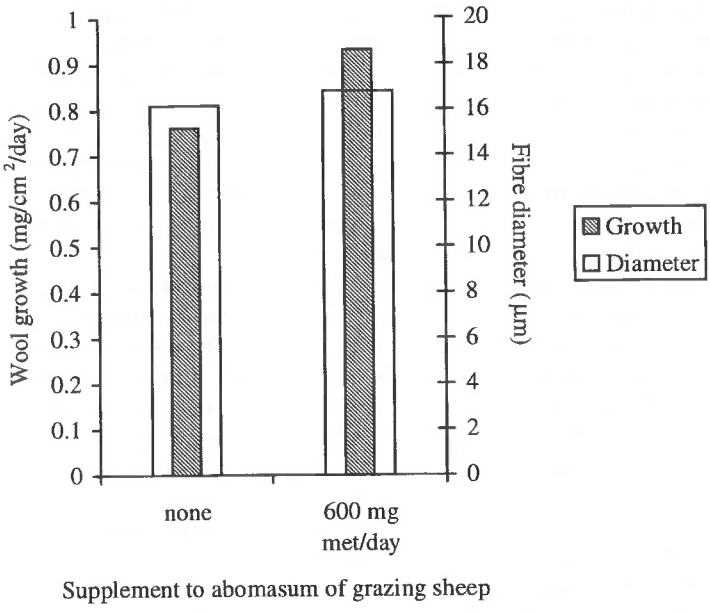


*Fig. 2.* Effects of methionine on wool growth and fibre diameter. Modified from Pickering and Reis (14). Sheep received supplements in the form of gelatin capsules via an abomasal cannula once daily. Wool growth was measured by clipping a midside area of about 100 $cm^2$, at three-week intervals. Values given are the means for 5 animals. Maximum effective standard error was 1.25 mg $cm^{-2}$ $day^{-1}$. Fibre diameters are the means for 5 animals. Maximum effective standard error was 0.75 mm.

We have identified proteins that are both rich in the sulphur amino acids, and naturally resistant to degradation in the rumen (15). One of these, sunflower seed albumin 8 (SSA), which contains 23.3 percent methionine plus cysteine (4), was degraded from a polypeptide of 12 kilodaltons, with a half-life of three hours, to a polypeptide of 8 kilodaltons that persisted in an *in vitro* rumen experimental system with a half-life of 69 hours (16). SSA has therefore been the basis for an approach designed to supplement pasture legumes with rumen-protected sulphur amino acids, via expression of introduced genes in transgenic plants.

We constructed a chimeric gene incorporating the protein-coding information for SSA fused to a strong, constitutive plant promoter from the *35S* gene of cauliflower mosaic virus (CaMV). The *35S-ssa* gene was transferred to tobacco and lucerne using *Agrobacterium tumefaciens*-mediated transformation. A number of transgenic plants were generated in each case, and northern blotting of total leaf RNA showed the presence of readily detectable levels of SSA mRNA in most transformants. However, no SSA protein was found in the leaves of the transgenic plants, indicating a post-translational limitation to protein accumulation (17). Dicot seed storage proteins contain targeting information which directs their intra-cellular transport from the endoplasmic reticulum (ER) to the vacuole of the developing seed. In this organ, the vacuole is specialised for protein storage, whereas the function of the vacuole in leaves is thought to be analogous to that of the hydrolytic lysosome of mammalian cells. It was concluded that the SSA protein was unstable in its vacuolar location in leaves (17), as had been previously observed for a pea seed storage protein, vicilin (18).

It was demonstrated in the case of vicilin, that re-direction of the protein to different subcellular locations resulted in increases in the half-life of the protein, and in the consequent accumulation of the protein in leaves. The protein was found to accumulate to the highest levels when targeted to the ER (18). Similarly, when the *ssa* gene was modified to include a nucleotide sequence encoding a carboxy-terminal ER retention signal (lysine-aspartate-glutamate-leucine, KDEL, see Fig. 3a), the SSA protein was readily detectable in leaves of transgenic tobacco and lucerne. However, the maximum levels of SSA expression in lucerne were estimated to be too low to result in significant nutritional enhancement of the transgenic plant material (15, 17).

The *35S-ssakdel* gene was also transferred to subterranean clover, the major pasture legume grown in southern Australia, using an *A. tumefaciens*-mediated transformation system (19). A progeny line homozygous with respect to the transgene was selected from the transgenic plant expressing the highest level of SSA. Under glasshouse conditions, the sunflower albumin accumulated in progressively older leaves of the transgenic plants, indicating that SSA was more stable than the average leaf protein (Fig 3b). SSA was estimated to comprise 0.3% of the total extractable protein of young leaves, increasing to approximately 1.3% in the oldest leaves, with an average over the aerial parts of the plant (leaves and stems) equal to 0.75% of total extractable protein.

It has recently been demonstrated that the above levels of SSA expression are maintained under field trial conditions. Assuming that the dry matter of subterranean clover is 20% protein, it can be calculated that a sheep consuming 1 kg of dry matter per day would ingest approximately 1.5 g of SSA, containing approximately 300 mg of rumen-protected methionine plus cysteine. This level of sulphur amino acid supplementation is at the lower end of the range of levels reported to result in significant increases in wool growth rate in sheep. Langlands (20) recorded an increase of 23% in wool growth in sheep supplied 360 mg of methionine per day direct to the abomasum, while Pickering and Reis (14) reported a 22% increase in wool growth in response to 600 mg of methionine per day supplied via an abomasal cannula (see Figure 2).

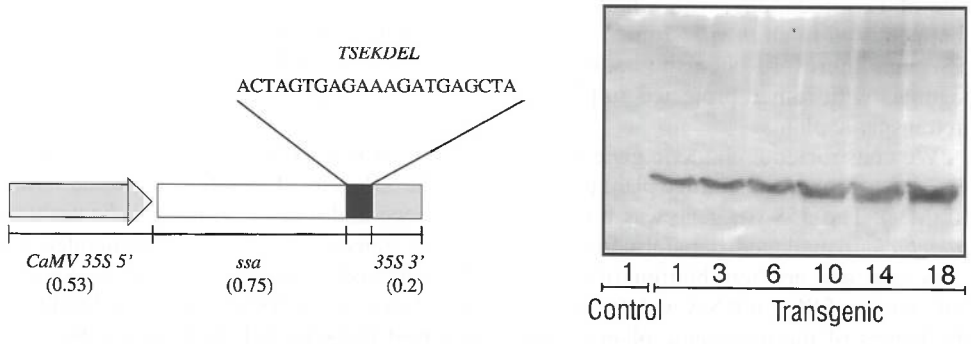


*Fig. 3.* Expression of SSA in transgenic subterranean clover.

a) Structure of the *35S-ssakdel* gene. Gene construction was as described in reference 17. The stop codon of the SSA protein-coding region was replaced with the nucleotide sequence shown, encoding a seven amino acid extension, which ends with the ER retention signal. Numbers in brackets refer to the lengths of DNA fragments in kilobases.

b) Accumulation of SSA in progressively older leaves of transgenic subterranean clover. Modified and reproduced with permission from reference 19. A western blot with 40 μg total leaf protein per lane, probed with antiserum to SSA. The numbers refer to the leaf number, starting with the youngest leaf, from control or transgenic subterranean clover plants grown in the glasshouse.

Given that subterranean clover usually constitutes 50% or less of the diet of grazing sheep, we would predict that sheep would obtain an additional 150 mg of cysteine and methionine from the transgenic subterranean clover in a field situation. We therefore aim to increase the level of SSA in transgenic subterranean clover by expression of modified SSA-encoding genes in the transgenic plants. It should be noted that increases in wool growth rate resulting from sulphur amino acid supplementation are accompanied by increases in wool fibre diameter (14). Too large an increase in fibre diameter will result in a reduction in the quality of the wool. Therefore our ultimate aim is to achieve relatively modest increases in wool growth rate, accompanied by only slight increases in fibre diameter. For example, as shown in Figure 2, Pickering and Reis (14) recorded a 22% increase in wool growth accompanied by only a 3.7% increase in fibre diameter.

**Adding proteins rich in sulphur amino acids to the seeds of pulses**

The pulses, lupin, pea and chickpea are widely used as sources of protein in human and animal diets. The seed protein of pulses is regarded as being of high nutritional quality because of its relatively high content of lysine (around 4.5 to 7% of total protein). The limitation to utilisation of pulse seed protein lies in the low content of the sulphur amino acids (around 2.2 to 3% of total protein) which is well below the estimated dietary requirement of non-ruminant animals (approximately 5% of total protein). In order to supplement the seed protein of pulses with extra cysteine and methionine, we have constructed and transferred seed-specific chimeric genes encoding SSA to the narrow-leaf lupin, field pea and chickpea.

A chimeric gene was constructed incorporating the promoter from a pea vicilin gene (*vic*), which is normally expressed only in the developing pea seed between approximately 11 and 21 days after flowering (21). The vicilin gene promoter was fused to the DNA encoding an unmodified SSA protein (Fig. 4a). The gene was transferred to the three pulse species using *A. tumefaciens*-mediated transformation protocols established recently in our laboratory (22, Molvig et al, unpublished). In all cases, transgenic lines expressing readily detectable amounts of SSA in seeds were produced (Fig.4b).

A transgenic lupin progeny line (from cv Warrah) which was homozygous with respect to the transgene, was estimated to contain SSA equivalent to over 5% of extractable seed protein. The physical composition of grain from this line was compared with that of control, non-transgenic lupin seed (Molvig *et al*, in preparation, summarised in Table 1). Seed weights and total protein content did not differ between the two samples. The sulphur in each seed sample was estimated by an X-ray spectrometry technique that can be used to resolve two major fractions corresponding to sulphate (oxidised sulphur) and sulphur amino acids (carbon-bonded sulphur) in plant material (23). The total sulphur content in each sample was very similar, however the partitioning of that sulphur between the oxidised and carbon-bonded fractions was markedly different between the two samples. In the transgenic grain, the carbon-bonded sulphur fraction represented 79.6% of the total seed sulphur, in contrast to 62.7% in the case of the control seed. This equated to an increase in carbon-bonded sulphur of approximately 27% in the transgenic seed. Analysis of amino acid composition of the two samples showed a more than 100% increase in the methionine content of the transgenic seed. As far as can be assessed under glasshouse conditions, seed viability and seed yield of the transgenic plants was not altered in comparison to controls.

A rat feeding trial was performed with the transgenic lupin seed, and it was found that the increased sulphur amino acid content correlated with improved efficiency of utilisa

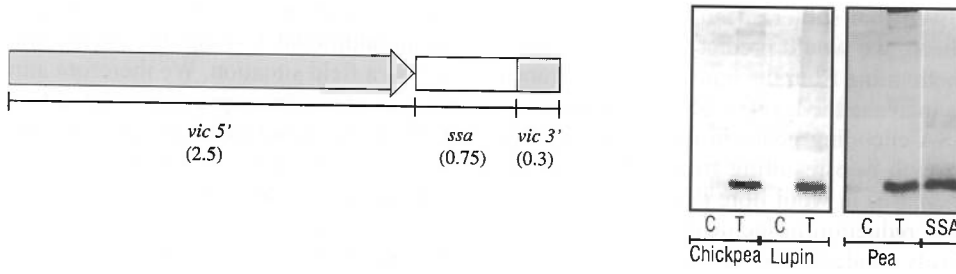


*Fig. 4.* Expression of SSA in seeds of transgenic chickpea, lupin and pea.
a) Structure of the *vic-ssa* gene. The numbers refer to lengths of DNA fragments, in kilobases.
b) Detection of SSA in seed extracts from transgenic pulses. A composite of two western blots showing; on one blot, the presence of SSA in 1µg of total protein from a single control (C) or transgenic (T) seed of chickpea or lupin, and in the other, SSA in 20 µg of protein from a single control (C) or transgenic (T) seed from pea, as well as 50 ng of pure SSA (SSA).

*Table 1.* Characteristics of transgenic lupin seeds

| Determination | Non-transgenic lupin | Transgenic lupin |
|---|---|---|
| Average seed weight (mg) | 157 | 154 |
| Protein (% DW) | 34.3 | 35.8 |
| Sulphur Total (ppm) | 3800 | 3900 |
| Oxidised (ppm) | 1411 | 790 |
| Carbon-bonded (ppm) | 2350 | 3085 |
| Methionine (g/16gN) | 0.65 | 1.40 |
| Biological Value (%) | 69.9 | 77.6 |

tion of the seed protein, as measured by biological value of the diet (Table 1). We have therefore demonstrated the feasibility of improving the nutritional quality of plant protein by addition of an exogenous protein rich in amino acids often present in limiting quantities in the diets of ruminant and non-ruminant animals. An SSA-containing lupin line is currently undergoing field trial to investigate its agronomic performance and to obtain large enough seed stocks for feeding trials with pigs, chickens and sheep. Transgenic field peas and chickpeas expressing SSA are also being evaluated.

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# CEREAL STORAGE PROTEINS: CHARACTERISTICS AND RESPONSES TO NUTRITION AND ENVIRONMENT

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## Abstract

Recent studies implicate the proteins of cereal grains in both the baking quality of wheat flour and also in the malting quality of barley. High molecular weight polymers between HMW prolamins and S-rich prolamins occur both as a critical component determining the properties of gluten in dough and have also been detected in extracts of the grain protein from malting barley. Two types of structural features have been identified within the sequences of these proteins which contribute significantly to the formation and properties of high molecular weight aggregates or polymers in both wheat and barley protein extracts. In both cases the properties of these aggregates are determined both by inter and intra chain disulphide bonds and also by the special secondary structures formed by the regularly repeating amino acid sequences which are characteristic of these proteins.
The impact of climatic and nutritional factors on the composition of the grain proteins in both wheat and barley is described. High molecular weight prolamins, the HMW glutenins in wheat and the D hordeins in barley, show particular sensitivity to growth conditions. In wheat, alterations to the proportions of S-rich prolamins and HMW glutenins induced by alteration in sulphur and nitrogenous fertiliser regimes have been linked to changes in specific parameters of baking quality. Evidence is presented that D hordeins, the HMW prolamins in barley, are similarly responsive to growth conditions, both N fertilisation and climatic, and that these proteins appear to play a key role in determining malting quality through their effects on cell wall modification. The development of molecular tools to manipulate these proteins is also discussed.

## Introduction

Cereals are the most widely grown crops in the world and produce close to 2000 million tonnes of grain per year (1). Since most of this grain is used to provide dietary protein for both humans and their livestock, cereal proteins have a long history as topics for research, stretching back to 1745 when the separation of wheat flour into starch and gluten was first described (2). This review will concentrate on the recent studies that implicate grain protein in both the baking quality of wheat and also in the malting quality of barley.

Whereas it has long been obvious that the physical properties of the proteins in wheat flour contribute to the ability of the flour to be baked into leavened bread, more recent research suggests that grain protein also impacts directly on the efficiency of the malting of barley. Malting and brewing, a rapidly expanding end use of barley in the Asian and Pacific area, requires the efficient hydrolysis, solubilisation and extraction of the endosperm of the grain. Current markets are tuned to the expectation of malt extract values of

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79 to 81%: that is, the malting process needs to lead to extraction of > 80% of starch from the endosperm as water soluble carbohydrate. Prices are set accordingly.

Despite its economic importance, malt extract is highly sensitive to both nutritional and environmental conditions during crop growth. The impact of nutritional and environmental factors on malting quality are considered below and recent studies on the role of particular grain proteins are described.

## Cereal Protein Structures

The types of cereal proteins that affect both baking and malting quality are the prolamin storage proteins. As major stores of grain sulphur, they are also the cereal proteins of most significance to this workshop. Prolamins are by definition a solubility class of proteins which are extractable from cereal grains in 50% to 70% alcohol (3). The name is derived originally from the relatively high content of proline and glutamine (3). Prolamins are only found in cereals and have been detected in all the major cereal crops of the world. With the exception of oats and rice, they are the major storage proteins present and constitute 40 to 50% of total grain N. Included in the prolamin class are the zein proteins from maize, avenin from oats, glutenins and gliadins from wheat, secalins from rye and the hordeins from barley.

This review will concentrate particularly on the prolamins from wheat, rye and barley. The major features of these prolamins as reviewed by Shewry (5) are as follows: amino acid sequence and DNA sequence data show that the prolamins from the Triticae are particularly closely related. The prolamin family within each Triticae species is further subdivided into three distinct groups, each with separate roles in the functional properties of the grain. The three groups of proteins which have been distinguished are the HMW prolamins, Mr > 80,000, the S-rich prolamins, Mr 45 to 70,000 and the S-poor prolamins, Mr < 45,000. Proteins within these groups also have common names based either on electrophoretic mobility or on the ability to form polymers. For example, prolamins in wheat are divided into the gliadins which are the monomeric proteins and the glutenins which are the proteins which form polymeric aggregates.

The molecular structures of these proteins have been studied to determine the contribution of particular prolamins to the properties of gluten. Gluten is the viscoelastic mass which can be isolated by washing dough to remove starch and water soluble compounds (4). It is the protein complex which forms the networks within doughs essential for bread and pasta making.

Whether from wheat, barley or rye, the prolamins are divided into three distinct types coded by separate gene families, whose essential features are outlined Figure 1.

Each type of protein is apparently derived from a common ancestral gene originally containing the regions A, B & C and modified by the inclusion of repeat regions where the protein structure contains short peptides, from penta to octapeptides, repeated many times (41). Each repeat region is characteristic of the particular protein but all repeats share a number of common features.

(i) They are all proline/glutamine rich repeats.
(ii) In general they appear to form regularly repeated secondary and tertiary structures dominated by repeated β-turns (6).

Computer modelling using the Chou and Fasman equations (7) predicts frequent β-turns within the repeat regions of HMW glutenins and similarly frequent β-turns within the repeat region of C hordein, the sulphur-poor prolamin from barley, due to the presence

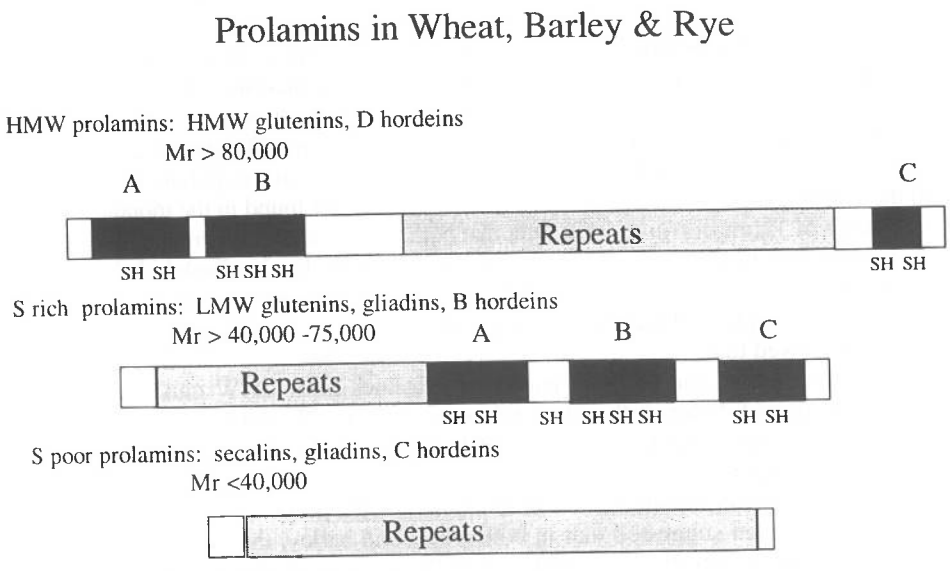


*Fig. 1.* Prolamins in Wheat, Barley and Rye: Protein sequence domains characteristic of HMW, S rich and S poor prolamins respectively (modified from Shewry (5)). Regions A, B & C correspond to three domains from a common ancestral protein as postulated by Shewry & Tatum (41).

of the repeated sequence Phe Pro Gln Gln Pro Gln Gln Pro (8). The repeat regions of sulphur-rich prolamins are similarly rich in potential β-turns. Both the modelling studies of Kasarda *et al.* (9) and the images obtained with scanning tunnelling microscopy of HMW glutenin molecules in the hydrated solid state suggest that these β-turns in the repeat regions tend to take up a loose spiral structure. The images obtained for pure HMW glutenin from wheat suggest a structure which is an array of aligned molecules with diameters of 19.5A and with diagonal striations consistent with a spiral structure with a periodicity of 14.9A (10).

Similar SAXS images which have been obtained with the S-rich and S-poor prolamins highlight the importance of the repeat regions in determining the shape of these proteins (11). The ultimate model for the HMW prolamins in cereals takes the form dominated by the 480 to 680 residues long spiralling repeat region. This is flanked at the amino and carboxyl terminal ends by less organised globular regions dominated by α helical secondary structure (12). In the case of the S-poor prolamins, the C hordeins and ω-gliadins, where >90% of the sequence is repeat region, the properties of these proteins are likely to be similarly dominated by the properties of the loose spiralling structure of these repeats (13, 14, 15, 16).

In addition to the repeat regions, the sequences of the sulphur-rich prolamins and the HMW glutenins also contain non-repeat regions including the A, B and C domains that have been proposed as relics of a common ancestral protein (Fig. 1). Most of the cysteinyl residues in these proteins are located within these ancestral domains. Where they have been retained in the modern proteins they add an extra and key functional property to these proteins. The cysteinyl-rich regions of the proteins contribute in two ways to the functional properties of these proteins. They contribute to both intra and inter molecular disulphide bonding within and between protein chains. The sulphur-rich prolamins have been studied in some detail in this regard.

As shown in Figure 2, the sulphur-rich prolamins can be divided into two functional types according to the proposed disulphide bonds between the cysteinyl residues (4, 17). Firstly, there are the monomeric gliadin molecules, represented here by the α & γ gliadins, where it is considered the cysteinyl residues participate only in intrachain disulphide bond formation as shown. The resultant molecules form only monomers.

By contrast, the LMW subunit of glutenin, which exists *in vivo* in polymeric complexes with the HMW glutenins, contains two cysteinyl residues not found in the monomeric gliadins. Studies of Thompson *et al.* (18) infer that both of these cysteinyl groups, those shown as free sulphydryl groups in Figure 2, are involved in intermolecular disulphide bonds.

Thompson *et al.* (18) replaced either one or both of these cysteinyl residues by genetic engineering and expressed the resultant sequences in *E. coli.*
The results showed that:

(i) Deleting either one of these particular cysteines from LMW glutenin led to the production of dimers rather than polymers of the LMW glutenins.
(ii) Deleting both cysteines from the LMW glutenin produced only monomers of the LMW glutenins.
(iii) Deleting neither resulted in the production of some polymers.

*In vivo* it has been suggested that in both wheat and barley, the interchain disulphide bonds include bonds between the HMW and LMW subunits of glutenins leading to models for polymer structures as proposed by Moonen *et al.* (19). Evidence in support of the theoretical model has been obtained in the last three years by studies with size exclusion chromatography of the gluten polymers, extracted from flour without disruption to disulphide bonds, by peptide mapping (20, 21) and by the use of genetic engineering (18, 22). These studies are finally beginning to assign the links in the proposed polymer structure to particular cysteinyl residues.

The properties of the prolamin polymers established in wheat (6, 23, 24) may be summarised as follows. *In vivo* gluten occurs as polymers of molecular weights approximately 1 to 10 million containing three to five HMW glutenin subunits and up to forty LMW

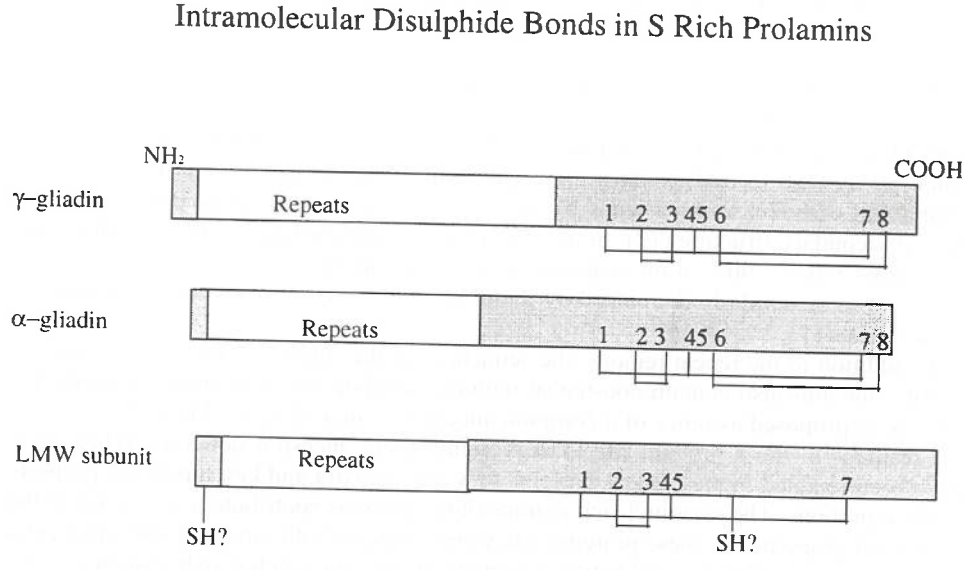


*Fig. 2.* Intramolecular Disulphide Bonds In S Rich Prolamins. Reproduced from Thompson et al.(18).

glutenin subunits held in an aggregate by specific disulphide bonds. When gluten is extracted from flour by simply washing away the included starch and water soluble protein, the proteins appear to exist as very high molecular weight polymers. The ability to form these high molecular weight polymers has been linked to baking quality. Studies with infrared spectroscopy of gluten complexes has shown that in the dough-like state, the complexes contain much higher proportions of intermolecular β-sheets than in the individual subunits in solution (25). Interactions between subunits in glutenin polymers and aggregates which involve these intermolecular β sheets located in the repetitive domains are considered to be responsible for the viscoelastic properties of gluten proteins (26). NMR spectroscopic studies on pure HMW subunits of glutenin showed that disulphide bonds were also important in determining the extent of the β-sheet formation (27).

## Wheat Quality vs Sulphur

The very few studies which have been carried out on the effects of sulphur nutrition on baking quality also implicate the HMW/LMW glutenin aggregates. Of particular interest to the conference is the work of MacRichie & Gupta (28) who re-examined the nature of flour samples generated in wheat in an Australian nitrogen and sulphur fertiliser response trial (29, 30). As shown in Figure 3, Olympic wheat grown under a range of nitrogen and sulphur regimes produced flour samples with a wide range of baking qualities.

Protein composition was certainly affected by growth conditions in this trial (29). The data shows that higher sulphur in the grain led to the formation of higher contents of gluten polymers in the flour and to associated alterations in baking quality. For example, as shown in Figure 3, the extensibility of the dough was directly correlated with % flour polymeric protein, the measurement used in this study to estimate gluten polymer forma-

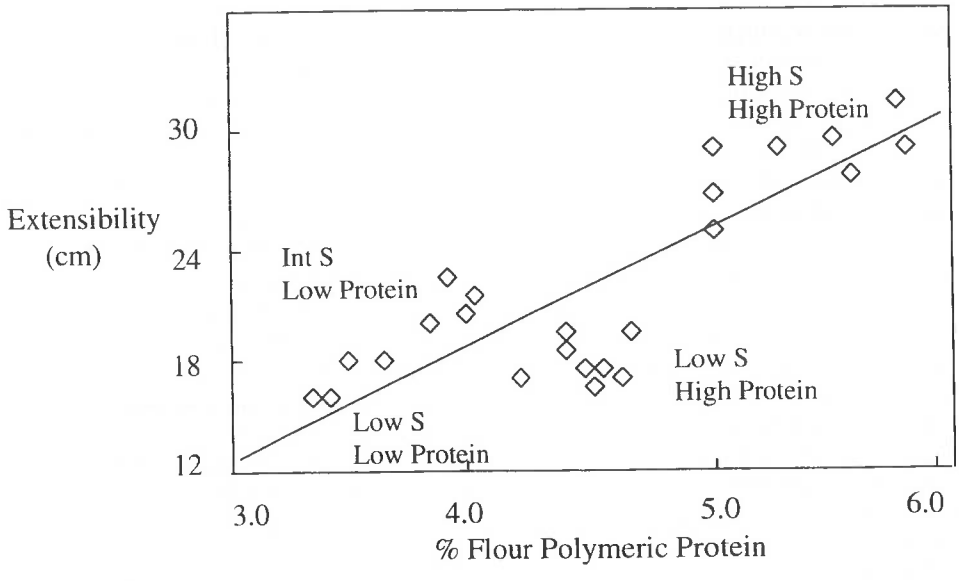


*Fig. 3.* Effects of Protein and Sulphur Contents on Extensibility and Polymer Content in Wheat Flour. Reproduced from MacRichie & Gupta. (28).

tion. Dough extensibility varied over almost a three-fold range with the high sulphur, high protein samples grouped with the highest extensibilities and with mid and low sulphur samples grouped at the lower extensibilities.

Wrigley *et al.* (30) showed that one of the primary influences of sulphur deprivation on grain composition was to change the ratio between S-rich prolamins (LMW glutenins) and the HMW glutenin subunits. HMW glutenins were synthesised in preference to the more sulphur-rich LMW glutenins and gliadins. MacRichie and Gupta also showed that the ratio of HMW/LMW subunits in the gluten correlated with the % of high molecular weight gluten polymers in the above flour samples. Haneklaus and Schnug (31) demonstrated the extent to which sulphur nutrition similarly affected wheat quality in European crops and concluded that environmental factors were more important than cultivar in determining grain S concentration. So even though there are relatively few detailed and specific sulphur response trials, it is clear that HMW/LMW ratios in wheat are responsive to the environment and to the agronomic practices affecting crop growth and when they respond there can be big effects on baking quality.

The extensive data on genetic manipulation of glutenin subunit composition will not be covered in this review even though it too confirms the importance of HMW aggregates of prolamins in determining baking quality. Instead evidence will be presented which suggests that a major effect of the environment and of agronomic practices on the malting quality of barley is also via effects on the protein composition of barley grain, especially on the HMW prolamin fraction.

## Malting Quality in Barley

Barley is the second major crop where S-rich prolamins have a major economic impact. Barley suitable for malting receives a considerable price premium over feed-grade barley. The process of malting barley to provide the starting material for the brewing industry requires the germination of the grain for four days to break down the grain structure sufficiently to allow a high level of extraction of the endosperm contents. Barley is sold on the basis of a high malt extract, that is, a high percentage of endosperm contents solubilised.

There are two barriers to successful malting. Malting needs grain to absorb water first, then to secrete hydrolytic enzymes from the aleurone and scutellum layers into the endosperm (32). Physical access of these hydrolytic enzymes to the contents of the endosperm is potentially blocked by both the β-glucan in the cell walls and the proteins in the endosperm. Both these barriers need to be penetrated to achieve successful malting (32).

In Australia the climatic conditions at the critical stages of grain growth in the late spring and early summer can be variable. Hot dry finishes to the season occur regularly. There is great interest in the impact of such growth conditions on malting quality and it has been known for some time that such hot dry finishes often lead to excess protein accumulation and to poor malting quality. The studies outlined in the remainder of this review implicate the HMW and S-rich prolamins in such environmentally induced changes in malting quality. The studies which come largely from my own laboratory suggest that environmental impact on the HMW/LMW prolamin families of proteins in barley grain contributes significantly to the impact of growth conditions on the malting quality of that grain. That is, HMW/LMW prolamin ratios are important not only for baking quality in wheat but also for malting quality in barley.

The impact of growth and climatic conditions was examined in a field study conducted at Horsham, Australia. Barley was grown in two seasons (one ideal and one with a hot dry finish), under five different regimes of added N fertiliser and across three cultivars

(33). N applications varied from sub-optimal to excessive. The impact of applied nitrogen and season on malt extract is shown in Figure 4.

1990 was a hot dry season while 1991 was milder and wetter. There was a major difference both within and between seasons on the quality of the malt and there were also differences between seasons in the responses of individual cultivars. e.g. *cv.* Schooner malted poorly in 1990 but very well in 1991. For many years it has been accepted that high protein content in grain is correlated with low malt extract (34, 35). As shown in Figure 5, the protein content of grain increased in the trial samples as applied nitrogen was increased.

While the higher protein contents achieved in response to added nitrogen fertiliser were associated with an expected trend towards lower malt extract in all cultivars, protein contents did not accurately reflect the large differences observed in malting quality for particular cultivars especially between seasons (Fig. 4, 5).

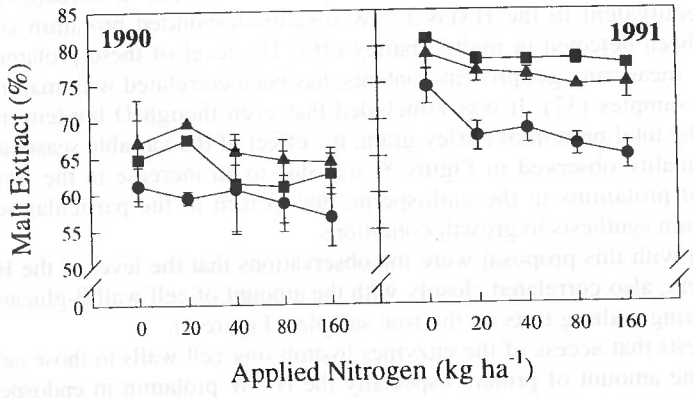


*Fig. 4.* Malt extracts for samples of barley from five nitrogen treatments for the cultivars, Arapiles (▲) Galleon (●) and Schooner (■) grown in two seasons (1990 and 1991). Error bars represent one standard deviation either side of the mean.

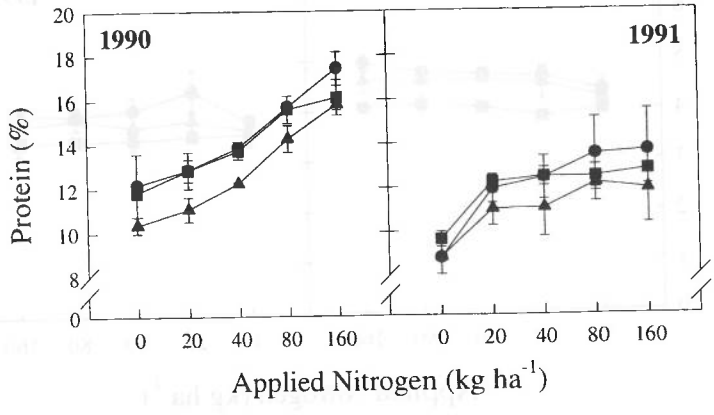


*Fig. 5.* Total protein content of barley grown in two seasons (1990 and 1991) using five nitrogen regimes and the cultivars Schooner (■), Galleon (●) and Arapiles (▲). Error bars represent one standard deviation either side of the mean.

The β-glucan content of the grain, the other potential barrier to successful malting, also did not appear to be the sole cause of the change in malting quality observed between seasons and between treatments. Figure 6 shows that the β-glucan levels in the grain varied little between nitrogen treatments and only slightly though significantly between seasons.

In addition to the total β-glucan and total protein contents of the grain, protein composition was also analysed. Major differences were induced in the protein composition of grain by the combination of growth conditions in the trial (Fig.7).

Figure 7 shows that there were major differences in the amounts of the various hordein fractions in grain grown under the different growth conditions. The content of D hordein in barley grain was particularly sensitive. It varied over a 10 fold range from 0.7 mg $g^{-1}$ to 7 mg $g^{-1}$ as shown in Figure 8, whilst total protein varied only 1.5 fold in the same grain samples. The level of D hordein was not only sensitive to N treatment. It was also particularly sensitive to season. When directly compared to malting quality, D hordein showed a high correlation ($r = -0.77$) with the key malting parameter, malt extract (Figure 8).

D hordein is the HMW prolamin of barley and D hordein /B hordein complexes or aggregates equivalent to the HMW/LMW disulphide-bonded prolamin complexes in wheat have been detected in malted barley (36). The level of these prolamin polymers, estimated by measuring gel protein contents, has been correlated with malting quality in other barley samples (37). It was concluded that even though D hordein made up less than 5% of the total protein of barley grain, the effect of the variable seasonal conditions on malting quality observed in Figure 8, was due to an increase in the level of HMW aggregates of prolamins in the endosperm, due in turn to the particular sensitivity of HMW prolamin synthesis to growth conditions.

Consistent with this proposal were the observations that the level of the HMW prolamin, D hordein, also correlated closely with the amount of cell wall β-glucan which was mobilised during malting tests on the trial samples (Figure 9).

This suggests that access of the enzymes hydrolysing cell walls to those cell walls was affected by the amount of protein especially the HMW prolamin in endosperm cells. A consequence of this was that when the effects of both the D hordein and β-glucan contents

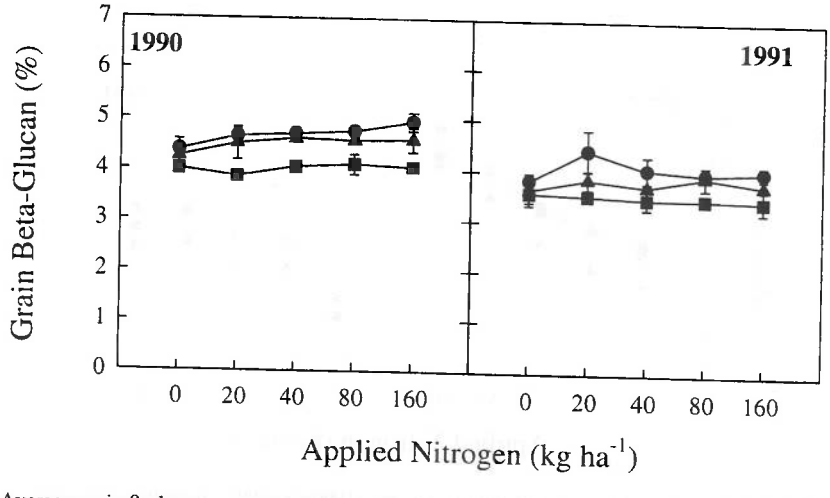


*Fig. 6.* Average grain β-glucan contents of samples from Galleon (■), Schooner (●) and Arapiles (▲) for each nitrogen treatment grown in 1990 and 1991. Error bars represent one standard deviation either side of the mean.

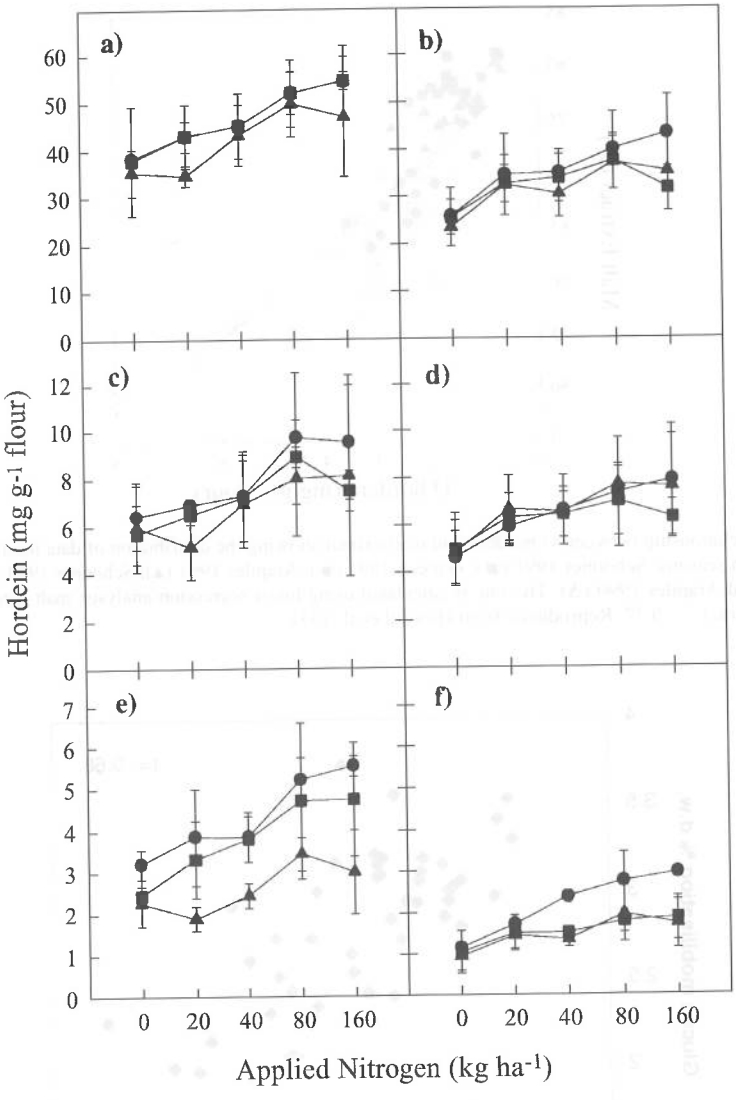


*Fig. 7.* Mean B hordein (a, b), C hordein (c, d) and D hordein (e, f) levels in barley grown at five nitrogen levels in two seasons, 1990 (a, c, e) and 1991 (b, d, f) for the cultivars Galleon (■), Schooner (●) and Arapiles (▲). Error bars represent one standard deviation either side of the mean.

of the grain were combined using a multiple regression analysis, it was possible to predict the potential malt extract of grain samples with a high degree of precision (Fig. 10).

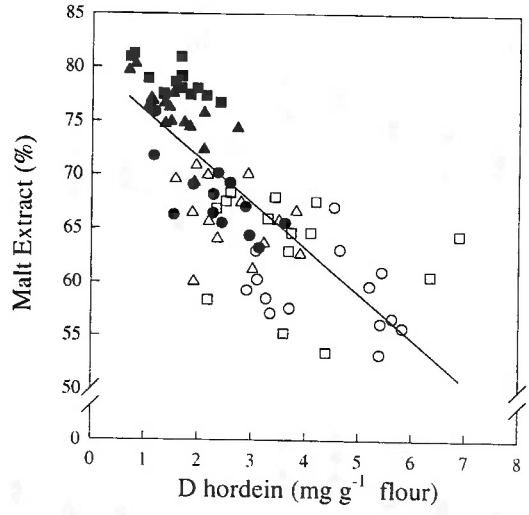


*Fig. 8.* The relationship between D hordein and malt extract showing the distribution of data for all three cultivars and both seasons; Schooner 1991 (■), Galleon 1991 (●), Arapiles 1991 (▲), Schooner 1990 (□), Galleon 1990 (●) and Arapiles 1990 (Δ). The line is calculated using linear regression analysis: malt extract = 80.1 – 4.23 D hordein, r = – 0.77. Reproduced from Howard et al., (33).

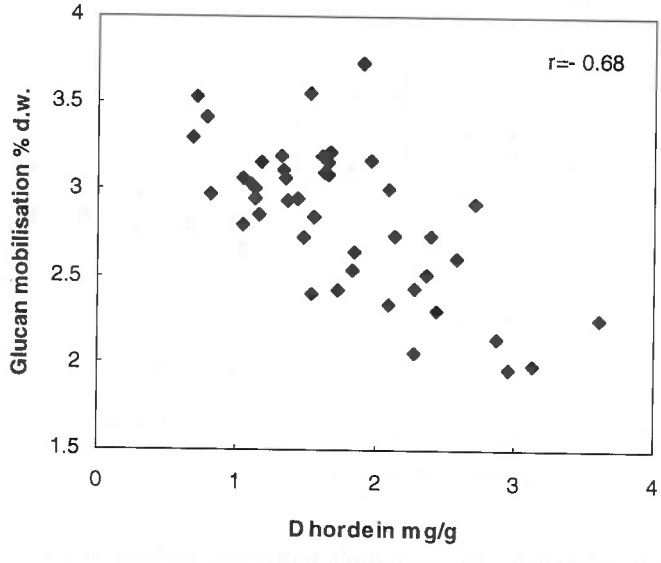


*Fig. 9.* Glucan Mobilisation in malted barley. Glucan mobilisation was estimated from the differences between β-glucan contents measured in flour before and after malting of all barley samples in Figure 8 and compared with the D hordein contents of the flour before malting.

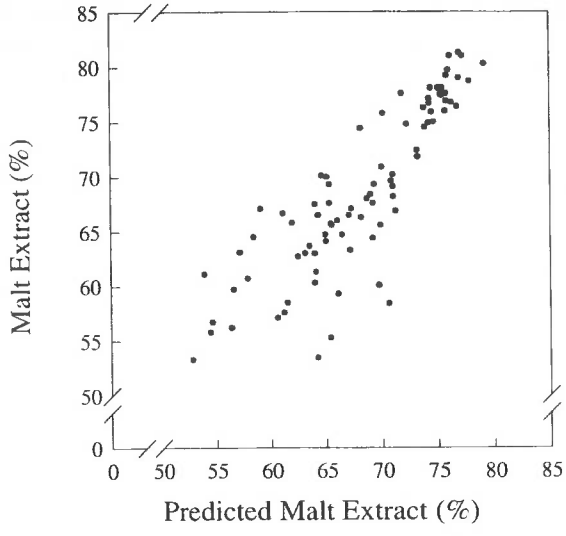


*Fig. 10.* The relationship between actual malt extract and predicted malt extract for samples from three cultivars grown under five nitrogen regimes and in two seasons. Predicted malt extract was calculated from the equation; malt extract = 108 – 2.71 D hordein – 7.93 grain β-glucan.

## Prospects for Genetic Manipulation of HMW prolamins in Barley

The prospects for genetic manipulation of the prolamins in barley as a means of modifying malting quality depend upon the complexity of the gene family encoding the prolamins in barley. The structures and sequences of the genes encoding HMW prolamins in barley have therefore been examined in my laboratory.

We have detected D hordein polymorphism. Different sized D hordein proteins ranging in apparent Mr from 85,000 to 95,000 were detected on protein gels. By using polymerase chain reactions (PCRs) based on the partial D hordein sequences from Halford *et al.* (38) and Sorensen *et al.* (39), we demonstrated that the protein polymorphism was due to differences in the length of the coding region of the genes. Seven different forms of the gene encoding D hordein have been detected and preliminary genetic analysis suggests that they are alleles at the *hor 3* locus. The seven different D hordein genes vary in length by multiples of approximately 30 base pairs and can be detected by a simple PCR based test. A similar amount of size variation in genes encoding the HMW glutenin subunits has been identified at the Glu-D1 locus in wheat by PCR (40).

The simple PCR based test for the D hordein genes is now being used to assist in the breeding of isogenic lines containing different D hordeins to determine the effect on malting quality of varying the size of the HMW prolamin in the grain.

## Conclusions

Three main conclusions can be drawn from the recent studies on HMW prolamins in barley and wheat.

Firstly, the amounts of the HMW prolamins in grain are particularly sensitive to nutritional and environmental influences during the growth of the crop.

Secondly, as a consequence of the alterations in the composition of the prolamins in grain both baking quality in wheat and malting quality in barley are altered.
Thirdly, it is now feasible to breed for particular HMW and S-rich prolamins and to target these for crop improvement.

## Acknowledgments

The assistance of J. Panozzo and A. Bedggood at VIDA, Horsham in the conduct and analysis of the major barley trial discussed in this work and the technical assistance of A. Bunnage with D hordein gene analysis is gratefully acknowledged. The research on the role of D hordeins and malting quality was funded by the Grains Research and Development Corporation, Australia.

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# SIGNIFICANCE OF SULPHUR FOR THE QUALITY OF DOMESTICATED PLANTS

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## Abstract

Though sulphur is one of the major plant nutrients the impact of an insufficient S supply on quality parameters of domesticated plants has been neglected in research as industrial S emissions have been excessive over the last century. Meanwhile, however, S is the most important limiting factor in European agriculture due to the desulphurisation of indistrial emissions. Sulphur deficiency has a direct influence on primary and secondary compounds such as protein content/quality and glucosinolates or alliins. The enrichment of potentially detrimental non-nitrogen compounds in sulphur-deficient plants is of special interest with view to vegetables especially as the quality in terms of phenological appearance is apparently higher in S-deficient plants. Among other technological features the baking quality is closely related to the S status of winter wheat. For wheat produced by organic farming sulphur fertilisation can contribute to maintain the baking quality of the grain as the use of improving agents is limited.
A better understanding of the natural defence systems of domesticated plants based on the release of $H_2S$ through the cuticle after root uptake of sulphate or the synthesis of secondray compounds like glutathione may contribute to a reduction of agrochemical input.
Last but not least the ecological relevance of a sufficient S-supply is revealed by its contribution to the degradation of surface ozone, non point nitrogen losses from agriculture and function of oilseed rape as a forage for honey bees.

## 1. Introduction

The domestication of plants has aimed not only at providing a secure supply of the very basics of man's life (energy, protein and fat), but also at providing spices for the improvement of food palatability, sources of distinct pharmaceutical value and other speciality products. Many of the substances which are responsible for particular quality aspects of plant products are sulphur containing compounds. Therefore it is no surprise that sulphur is the plant nutrient which has perhaps the strongest impact on plant quality. In addition to the direct relations between sulphur compounds and quality parameters, there are also indirect influences that need to be considered. Examples of indirect influences include the accumulation of nitrate and heavy metals in sulphur deficient tissue and greater susceptibility of sulphur deficient plants to abiotic and biotic stress factors.

Sulphur deficiency, mainly as a consequence of reduced atmospheric inputs, is becoming a common and frequent problem in crop production and it affects not only yield, but also product quality. There is no other nutrient supply in the agro-ecosystem that is as variable as the S-supply (82, 87). Therefore, most of the variability in quality parameters, as a result of S metabolism, is caused by environmental rather than genetic factors. This explains, for instance, why the same varieties of the same vegetable can have a different taste if they are grown at different sites.

*Sulphur Metabolism in Higher Plants, pp. 109-130*
*Edited by W.J. Cram et al.*

This contribution gives a comprehensive overview of the significance of various sulphur containing metabolites for plant quality from nutritional, pharmaceutical and technical points of view and their dependency on the plant's sulphur supply. It also emphasises some mechanisms by which sulphur metabolism may indirectly affect plant quality.

## 2. The significance of sulphur on the nutritional quality of domesticated plants

### *2.1 Direct impacts on plant quality*

#### 2.1.1 Protein content and protein quality

From an agricultural point of view the *total protein content* and *protein quality* of crops are important quality aspects.

Although partially genetically determined, the *total protein content* of crops shows a variability which can be traced back to nutritional factors, of which nitrogen is the most influential (36). There are many reports on the relationship between S supply and total protein content of crops in the literature, and these can be summarised as follows: if S is the only limiting nutritional factor, S deficiency will first result in a reduction of S containing amino acids in proteins (see below). As the amino acid composition of proteins is part of the genetic identity of a species, this will be a limited effect, after which reduced total protein concentrations will be the consequence of S deficiency (82, 97). In vegetative tissue, the boundary between alterations in amino acid composition and reduced total protein concentrations is indicated by the appearance of deficiency symptoms (85, 95).

Compared to vegetative material, the influence of S supply on total protein content in seeds is much more dependent on the species. Species with small seeds typically rely on oils and fats as the energy reserve for the embryo, e.g. the *Brassica* species. The total protein content of their seeds is very uniform and virtually independent of the S supply (92, 97). Adaptation of the metabolic sink to the S supply is maintained solely by the number of seeds produced by the plant (95). In species with larger seeds, it is necessary to differentiate between those with significant S-rich proteins in the endosperm and those with mainly carbohydrates as the energy reserve for the embryo. The latter, for instance *Zea mays*, will show only a small change in total protein concentration with variability in the S-supply. In comparison, species with large seeds and S rich proteins in the endosperm, for example *Vicia faba,* would adapt the quality of the endosperm proteins, to a certain extent, towards smaller amounts of S rich fractions when S becomes a limiting factor.

A major parameter determining the nutritional quality of plant proteins is the *content of essential amino acids*. These are termed "essential" because animals are not able to synthesise them and need a regular supply in the diet. Methionine is an example of a S containing essential amino acid and higher concentrations of methionine in plant proteins correspond to a higher biological value. The biological value is a relative figure used in nutritional science which scores the amino acid composition, in which egg yolk protein has a value of 100 and thus the highest score for quality. For a long time S supply has been identified as a major factor influencing protein quality (79, 80). Eppendorfer and Eggum (25, 26), for instance, found the biological value of proteins in potatoes reduced from 94 to 55 by S deficiency at high nitrogen supply and from 65 to 40 and 70 to 61 in kale and field beans, respectively. Whilst the essential amino acid concentrations declined due to S deficiency, amino acids of low nutritional value, such as arginine, asparagine and glutamic acid, increased (25, 26). Figure 1 shows the relationship between the S supply of curly cabbage plants, indicated by the total S concentration in fully expanded younger

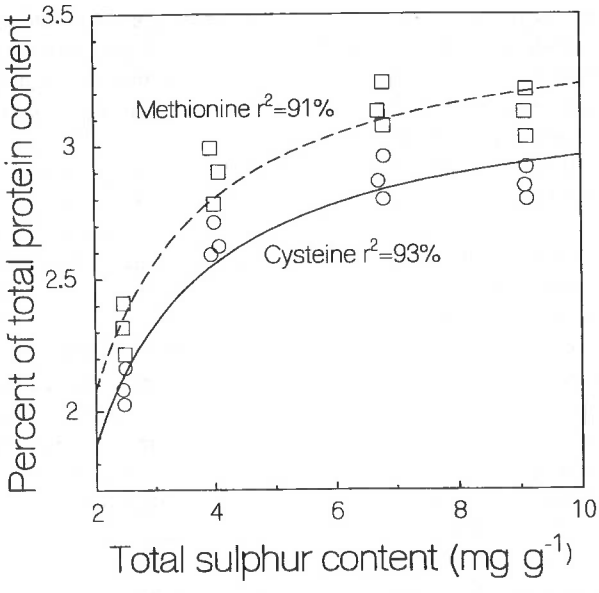


*Fig. 1.* Cysteine and methionine content in protein of younger, fully developed leaves of *Brassica oleracea* var. *sabellica* depending on the total sulphur content in the dry matter (85).

leaves, and the cysteine and methionine concentration in leaf protein. This example shows that a significant relationship between S supply and S containing amino acids exists only in conditions of severe S deficiency with visible symptoms. This level in *Brassica* species is below leaf S levels of 0.4 % total S in the dry matter (44, 95).

### 2.1.2. Sulphur containing secondary metabolites

Physiological functions and environmental relevance of S containing secondary metabolites have been extensively reviewed in the second book in this series (89). Their nutritional value is quite different from that of the products from primary metabolism. In many species, they are responsible for the characteristic taste of the plant, such as the glucosinolates in *Raphanus spp.* and *Brassica spp.*, the alliins in *Allium spp.*, thiazoles in *Lycopersicon spp.*, cyclic thiols in *Citrus spp.* and cyclic disulphides in *Asparagus spp.*

In the case of *glucosinolates*, however, there are two main antinutritive effects which are known. Both are in oilseed rape, which has become one of the most important oil crops in the northern European countries and Canada during the last 20 years. However, as the protein and glucosinolate containing residues from crushing are solely used for animal feed, both are only of interest in animal nutrition.

The first problem concerns a reduction in feed intake if the total glucosinolate concentration in the diet exceeds 10 μmol $g^{-1}$ (e.g. 41).

The second problem is the antagonistic effect between thiocyanate ions derived from the enzymic cleavage of glucosinolates in *Brassica* species and iodine anions in the thyroid gland of animals (23, 84). This effect is only important for monogastric species, as in ruminants all S containing compounds in the diet undergo microbial transformation in the rumen. It causes increased growth of the gland tissue (called the goitrogenic effect) to compensate for the reduced hormone output. In animal nutrition, the upper limit for

avoiding health problems from long term feeding on a glucosinolate containing diet is estimated to be 1 μmol $g^{-1}$ (54). The goitrogenic effect of glucosinolates has, however, never been observed in man, even in individuals with extraordinary high daily intakes, such as the famous philosopher Emanuel Kant who was known to consume large amounts of strong mustard every day.

Further information about glucosinolates, covering phytopathological and technical aspects of plant quality, will be given in section 2.2.3. and 3.2, respectively.

Glucosinolates occur in all parts of the plant with specific biochemical characteristics in secondary metabolism and contribute to the distinctive flavour or taste found in mustard seeds, horse radish roots or cabbage leaves. For further specific information on glucosinolates, refer to refs. 84 and 89.

Glucosinolate concentrations in plant tissues are highly variable and the most important source of variation is the S supply in the growth media. Genetically controlled variability is small in comparison (83). Moreover, the nitrogen supply, which has been suggested by some authors as an important source of variability, only indirectly affects glucosinolate concentrations via alterations in root growth. This affects the plant's access to soil S or the source/sink relationships in the plant. Additionally, alterations in root growth are only found in field trials and not in pot trials. More details about indirect effects of nitrogen on glucosinolates can be found in Schnug (86).

Figure 2 shows an example of the relation between S content and the total glucosinolate concentrations in curly cabbage leaves. In comparison to S containing compounds of the primary metabolism, which reach a saturation concentration if the S supply exceeds what is required for optimum growth (Fig. 1), S containing secondary metabolites increase linearly over a wide range of S supply. Examples for alliins are found in (50). Thus the formation of S containing secondary metabolites results in a by-pass of the back regulation of sulphate uptake. Compared to the total glucosinolate content, the pattern of individual glucosinolates, which is characteristic for each species, remains unaffected by variations in the S supply (70).

*Alliins* are S containing secondary metabolites giving *Allium* species their characteristic flavour and taste. In *Allium cepa*, they also cause the well-known lachrymatory effect brought on by cutting common onions (12). With alliins, as with glucosinolates, it is not the intact compound but the breakdown products of specific enzymic cleavage that show physiological activity.

As with glucosinolates, increasing S supply causes an linear increase of alliins in the tissue of *Allium* species. An example for *Allium cepa* is given in figure 2 from ref. 50, (this volume p. 331). However in *Allium* leaves, much greater amounts of S are bound to the alliins than to the glucosinolates of the *Brassica* species (compare fig 1 and 2 in ref. 50, this volume p. 331). The levels of S bound to glucosinolates in the vegetative tissue of *Brassica* species corresponds to what can be allocated to free sulphate (82). In comparison, the amounts of S in vegetative tissue of *Allium* species bound to alliins are one order higher. There is also no evidence that alliins are part of a dynamic storage system for S, as with glucosinolates, by which the plant is able to recycle S bound in secondary metabolites during phases of S deficiency (for more details see refs. 84, 89).

The alliins synthesized in the leaves are translocated into the bulbs immediately after they have been produced (20, 69). The S storage in the bulbs is then used by the regrowing vegetative shoot of the plant. It is always difficult to speculate on the reasons when it comes to secondary metabolites (6), but it seems as if the difference between the S storage strategy between species possessing glucosinolates or alliins has to do with their ecological survival strategy. Cruciferous crops like the *Brassica* species rely on a large number of relatively small seeds, which contain only the embryo and oil as energy back-

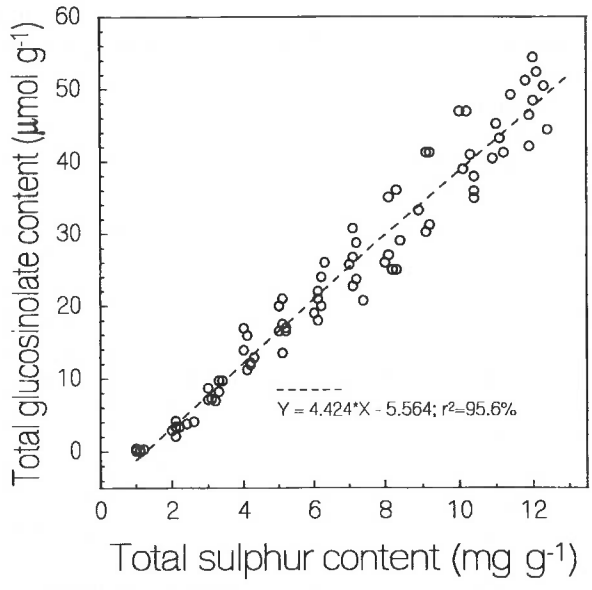


*Fig. 2.* Total glucosinolate and total sulphur content in the dry matter of younger, fully developed leaves of *Brassica oleracea* var. *sabellica* (compiled from 85).

up. Thus these seeds have a high content of S rich proteins and changes in the S supply affect the number of seeds per plant (95). Seeds of *Allium* species are even smaller but this species survives mainly via vegetative dissemination. This requires storage of S from season to season, rather than a short term accessible S reserve for seed production during phases of low supply, as in *Brassica* species. In other words, glucosinolates are a more dynamic storage pool than alliins.

Increasing content of S containing secondary metabolites with S supply results in the intensification of the taste and flavour of a particular crop. This explains why the same variety of the same vegetable or spice can taste or smell very different if grown on different sites. This does not necessarily mean, however, that quality is improved, as quality (in terms of taste and flavour) is very much dependent on individual consumer preferences. Strong cabbage or onion taste, for instance, may be attractive to one individual but not to another.

### *2.2 Indirect impacts on plant quality*

#### 2.2.1 Carbohydrates and fats

In general, S nutrition only has a small impact on carbohydrate and fat concentrations in crops.

There are three nutritional scenarios in which both are influenced by S supply:

1: If protein biosynthesis is reduced due to S deficiency, there will be an increase of glucose in the tissue which later will promote the formation of anthocyanides (95).

2: Carbohydrate and fat concentrations in seeds will tend to decrease if S fertiliser is applied to S deficient plants; this is simply a dilution effect by additionally synthesized proteins.

3: If S deficiency is limiting the development of an adequate plant canopy, rates of photosynthesis will decrease. This is of importance to root crops, such as sugar beet or potatoes, accumulating sugar or starch. In this scenario concentrations of carbohydrates are not necessarily altered but agronomic yields are decreased (37).

### 2.2.2 Non-protein nitrogen compounds

As S and nitrogen are closely tied up in amino-acid and protein biosynthesis, shortage in the S supply can influence the quality of plants indirectly by enrichment of non-protein nitrogen compounds like amides and nitrate.

Enrichment of amides is primarily a problem of excess nitrogen supply (101), but can be promoted if S deficiency occurs at the same time. Amides are known to cause an unpleasant "off-flavour" in vegetables. The most important non-protein nitrogen compound enriched following S deficiency is nitrate. Nitrate is prone to microbiologically induced reduction to nitrite during storage and processing of vegetables, and nitrite is toxic to humans by blocking the oxygen carrying capacity of haemoglobin (like carbon monoxide) or as a potential precursor for carcinogenic nitrosamines (22).

Figure 3 demonstrates the effect of S and nitrogen supply on the nitrate concentration in leaves of curly cabbage. Nitrite enrichments are to be expected if there is no sink for nitrogen in protein metabolism, either because the metabolism is hampered by S deficiency or it has reached its saturation (see also fig. 1). Nitrate accumulates with severe S deficiency or excess nitrogen supply (fig. 3). The highest levels are found if both events occur at the same time. Moreover, increasing the S-supply cannot prevent accumulation due to excess nitrogen supply (fig. 3). Further information on nitrates can be found in section 3.1.

### 2.2.3 Sulphur induced resistance

The contamination of domesticated plants with pesticide residues, via air- or soil-borne pollution of soils, reduces their nutritional quality. Consumers are becoming increasingly concerned about this problem and consequently markets for plant food from production systems avoiding such contamination (e.g. organic and biodynamic farming (60, 61, 81, 104, 107, 108)) are expanding.

It has been known, for a long time that sulphur has protective effects against pests and diseases. Most of the knowledge is, however, restricted to the external effects of foliar applied S (53, 56). Less is known about soil supplied S which has a strong influence on plant resistance by directly stimulating biochemical processes in primary and secondary metabolism. An example from field trials on an S deficient site in Scotland is given in ref 99, where a disease resistant oilseed rape variety and a non-resistant variety were treated with soil applied S and a foliar applied fungicide. The non-resistant variety showed a much greater response to the fungicide under S deficiency than the resistant variety did. Another example is the regional abundance of the light leaf spot disease which proliferates much faster in S deficient environments (90). It has also been shown that at insufficient S supply, higher pesticide inputs are economically justified (102, 112), which increases the risk of crop contamination.

All this indicates a special interaction between S supply and plant health but as with many other reports on the beneficial effects of S fertilisation on crop health (e.g. 73, 113), the results do not show causalities. Improved understanding of how S is involved in the stress resistance of plants, together with proper diagnostic methods and efficient fertilizer strategies could be a challenge in future production techniques. The aim should be to maximise the inherent potential stress resistance, which otherwise would not be

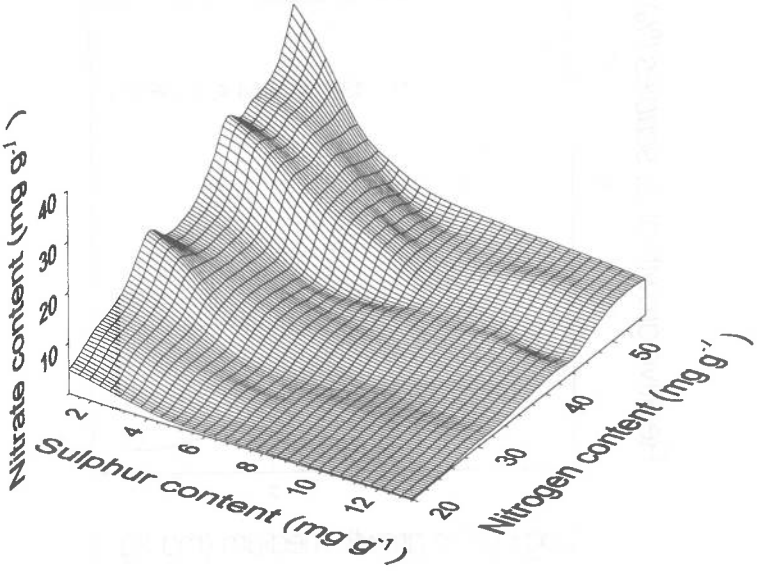


*Fig. 3.* Nitrate content in the dry matter of younger, fully developed leaves of *Brassica oleracea* var. *sabellica* depending on the total sulphur and nitrogen content (85).

expressed due to insufficient S supply, and help to maintain environmentally and economically sustainable farming.

In pathways prior to amino acid biosynthesis, the release of volatile S compounds is an important factor in S induced resistance: During the reduction of soil absorbed sulphate, significant amounts of S are released into the atmosphere, $H_2S$ being the predominant form followed by dimethylsulphide (100). The losses of volatiles compounds to the atmosphere amount to approximately 2-3 kg $ha^{-1}$ $y^{-1}$ for crop plants (1). Losses are proportional to the S supply (21) but are far too little to be understood as a regulatory step in order to level the S pools in the plants (77, 100). Although the amounts of S are too small to contribute significantly to the S balance of plants, an important but so far neglected effect of gaseous S emissions from plants could be their toxic activity against fungal attacks on the leaf surface. Examples for *Streptomyces scabies* and its infections on potatoes are given in figures 4 and 5. Therefore, it is likely that the flush of $H_2S$ during uptake by the reduction of the soil applied S helps the plant to combat fungal attacks.

Another mechanism by which S deficiency may promote fungal infections is an increased production of arginine which attracts certain fungi, like *Diplodia* and *Dothistroma* species, to pine trees (fig. 6) (65, 66) (see also 2.1.1.).

An indirect effect of the release of reduced volatile S compounds could be their reaction with surface ozone (see also 3.7) by which oxidative stress would be lowered even outside the organism.

*Glutathione* (GSH) is the major free, low molecular weight, non-protein thiol compound in the plant (10). It not only plays a role in storage and distribution of reduced S in plants but it is also known as an essential component of the plant's defence system against, for instance, oxidative stress (118) and for the detoxification of xenobiotics (42, 78). The positive effect of GSH on drought-, chilling- and freezing resistance, however, is still questioned (62, 63). S supply is a major factor maintaining the GSH content. As shown by the

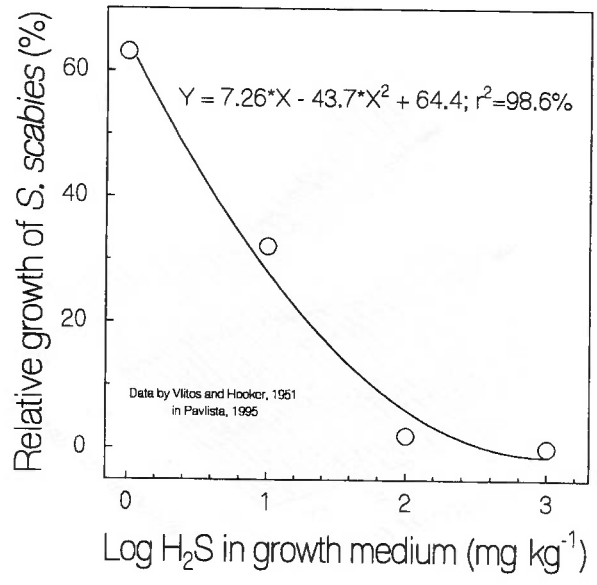


*Fig. 4.* Relative growth of *Streptomyces scabies* depending on the $H_2S$ concentration in the growth medium (compiled from Vlitos and Hooker, 1951 in (72)).

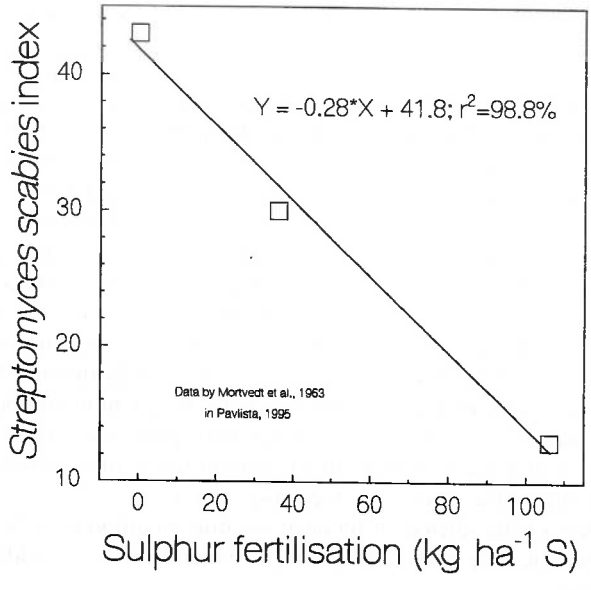


*Fig. 5. Streptomyces scabies* infections on potatoe tubers depending on sulphur fertilisation (compiled from Mortvedt et al., 1963 in (72)).

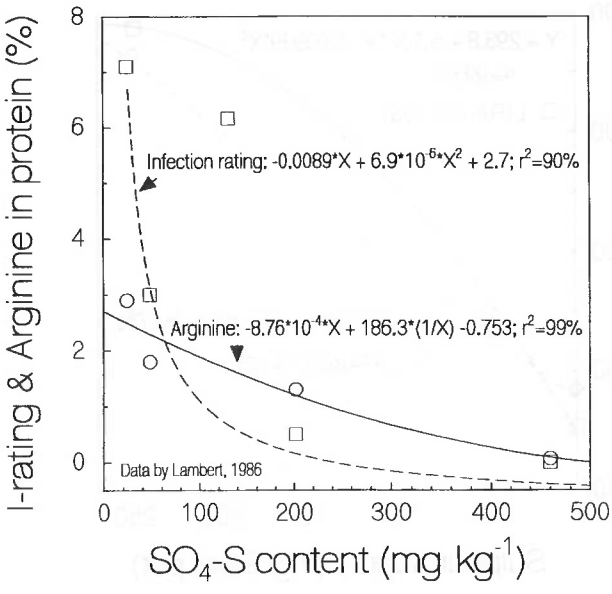


*Fig. 6.* Infection rating with *Dothistroma sp.* and sulphate content in the dry matter of *Pinus radiata* needles (compiled from (66)).

example in figure 7, S deficient plants have very low GSH concentrations whereas S fertilisation strongly increases the free thiol content. Thus S deficient plants should be more vulnerable to stress factors which are normally compensated by the GSH system and so S fertilisation should have a positive effect on resistance mechanisms provided by the GSH pathways. However, there is still much more experimental work needed to prove this.

Secondary metabolites, when present in a certain species, have always been a source of speculation on their impact on the natural resistance of the plant.

*Phytochelatins* are synthesised from glutathione by plants under heavy metal stress (78). The elements Ag, As, Bi, Cd, Cu, Pb and Zn have been shown to induce the synthesis of these compounds (39, 75). Heavy metal stress is an increasing problem for plants because metal loads in soils are continuously being raised due to the increasing recycling of wastes and sludges in agriculture and by long term fertilising with phosphates. As synthesis follows exposure, its not very likely that phytochelatins contribute to the heavy metal resistance of plants. Their indirect significance for nutritional quality is considered in 2.2.4.

Probably the most important ecological function of *glucosinolates* is to act as a storage for S via enzymic recycling (89). There has been much speculations about role of glucosinolates in the plant's defence system against pests and disease (28, 90). As the glucosinolate content is positively influenced by an increasing S supply, stress resistance mechanisms attributed to glucosinolate are enhanced too. The S containing *phytoalexins* of the *Brassica* family are synthesised as stress metabolites by cycling the thiocyanate component from indolglucosinolates following microbial infections or abiotic stress attacks (40). Recently these compounds have been understood to be defence chemicals. *S-Betaines* (3-dimethylsulfopropionate, DMSP) are known as effective osmoprotectants and have been identified in significant amounts in several members of the angiospermae (51). However, as with the phytoalexins, nothing is known about the environmental effects on variability.

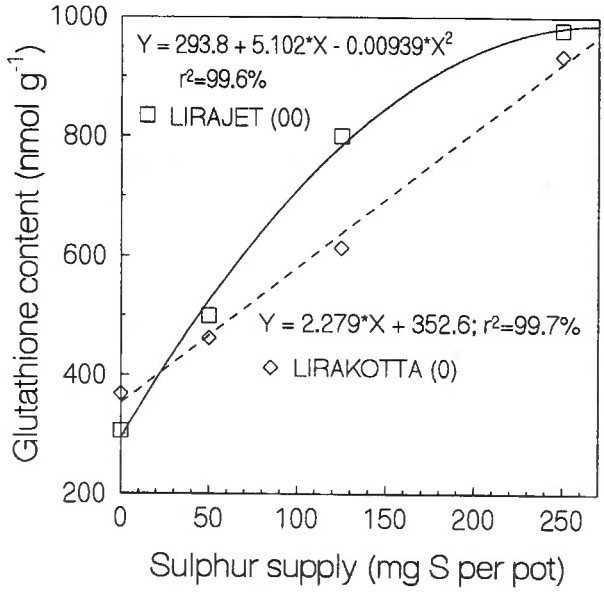


*Fig. 7.* Glutathione content in the dry matter of younger, fully developed leaves of single (0) and double (00) *Brassica napus* varieties depending on the sulphur supply (83). ["0" varieties are free of eruic acid, "00" varieties are also low in glucosinolates (see 84 and 89)].

The level of their dependency on the S supply may be speculated from the dependency of their precursors on S.

Enzyme inhibitors (e.g. of the Bowman-Birk family), lectins, thionins and γ-thionines are all cysteine rich proteins which are identified in several plant groups. These compounds seem to be part of the plant's defence system as they are highly toxic to microorganisms, insects and mammals. The first two groups of cysteine rich proteins are usually pre-synthesised, whereas the thionins seem to be newly synthesised after biotic stress attack (13). From the previously described relationship between common protein bodies and sulphur, an influence of S on quality and quantity of cysteine rich proteins can be assumed but still requires empirical proof.

### 2.2.4 Heavy metals

Excess heavy metal concentrations can seriously affect the nutritional quality of plants grown on contaminated soils. There are three ways by which S indirectly influences heavy metal concentrations in plants. The first is the increase of thiol concentrations in roots and stems, the second is the formation of *phytochelatins* and the third is the antagonism between sulphate and anions of heavy metals during root uptake.

The thiol group has a strong affinity to certain heavy metals, especially Hg, Cu and Cd. S deficient plants show low thiol concentrations in all plant parts which may promote the distribution of heavy metals within the plant. Alternatively, increased S supply, and therefore higher thiol concentrations, may result in higher levels of heavy metals being retained in the roots and stems of the plants (e.g. 3).

The formation of phytochelatins has been discussed as a specific interaction between S metabolites and heavy metals (see above). It is doubtful that, by producing phytochela-

tins, plants can protect themselves against heavy metal stress, but phytochelatins certainly reduce the transfer of heavy metals from roots to shoots or from stems to leaves, and thus prevent heavy metals from entering the food chain. It does not matter if this is due to heavy metal capture by the phytochelatin itself or simply by the previously described method of the binding of heavy metals to thiol groups. Reduction in copper concentrations, for instance, are found in shoots of S fertilised plants but increased levels are found in the roots (91). Increased lead uptake was reported to occur in S-deficient plants (125). Because the production of the precursor (GSH) is dependent on the S status it is likely that this mechanism is dependent on the S status as well. Thus S deficient plants may not be able to produce phytochelatins under heavy metal stress and, vice versa, S fertilisation may fortify the production of phytochelatins and limit the heavy metal uptake by plants.

Concentrations of heavy metals which are absorbed by plants as anions, such as arsenic, antimony, selene or molybdenum, are negatively affected by the S supply due to antagonistic competition during root uptake (91). This may have a positive effect on plant quality where plants on polluted soils are concerned, but under certain circumstances this may also lower the nutritional value by reducing concentrations of essential micronutrients (e.g. Se and Mo) for man and animals (37, 38, 68, 120). Additionally, in terms of plant nutrition, the antagonistic effect of sulphate on the uptake of molybdenum and boron can result in plant deficiency on marginal soils (93).

High S concentrations in the diet of ruminants are known to reduce the Cu supply to the animal, because the release of $H_2S$ by microbial activity in the rumen immobilises Cu by formation of insoluble CuS (71). However, as far as natural concentrations in plants are concerned, this effect is of little importance because the S concentrations above which there are significant effects on Cu-concentrations in animals, are much higher (Fig. 8).

## 3. Significance of sulphur for the technological quality of domesticated plants

### *3.1 Outward appearance*

Plant food often attracts consumers by its outward appearance, rather than by its nutritional value. Vegetables for instance are classified and marketed solely by their outward appearance (33). S deficiency has a great impact on appearance by directly influencing the colour and shape of leaves and fruits (9, 44, 95) but also by indirectly increased damage caused by pests and diseases (section. 2.2.3).

However, the effect of S deficiency on the appearance of leaves does not necessarily need to be a negative one. S deficient leaves are a lighter green to yellowish colour (44) which provides an illusion of younger age. At the same time, cell growth rates in S deficient leaves are reduced on the periphery but normal in areas along the veins. This causes mechanical distortion which gives the illusion of increased succulence and crispiness. In lettuce, for instance, both effects of S deficiency on colour and feeling result in higher value in terms of the appearance of the product. Unfortunately, these effects only occur with severe S deficiency and therefore nitrate concentrations will increase at the same time (Fig.9).

### *3.2 Seed quality*

Seed quality is not only determined by their nutritional value but also by their ability to provide the basis for the next harvest. S deficiency has no obvious influence on the fertil-

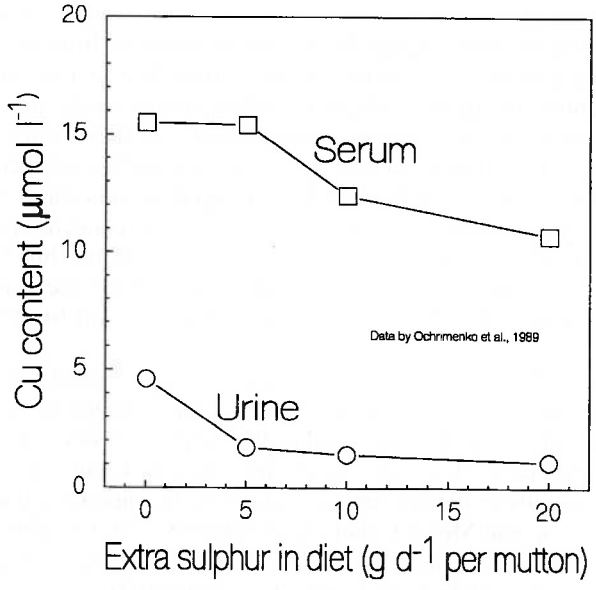


*Fig. 8.* Copper content in serum and urine of mutton as influenced by extra sulphur in the diet (compiled from (71)).

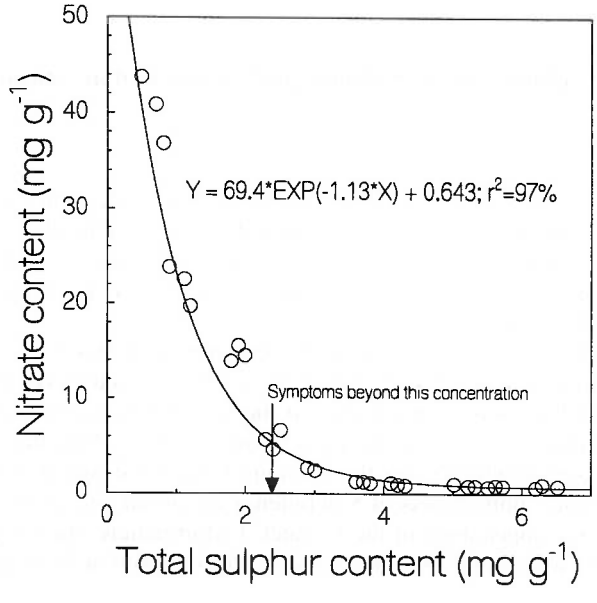


*Fig. 9.* Nitrate and total sulphur content in the dry matter of *Lactuca sativa* leaves (85).

ity of flowers but does affect yield components (48, 95). Plants with typically high seed weights, such as cereals and beans, have reduced seed weights with S deficiency and even greater deficiency causes the depletion of S containing amino acids in the protein fraction (section. 1.1.1). Both effects have a negative impact on seed vigour.

Seeds developed on S deficient oilseed rape plants are vulnerable to early germination in the pods prior to harvest, especially under humid conditions and when the harvest is delayed. The hypothesis explaining this phenomenon is the increased anthocyanine production under S stress (95) which has a stimulating effect on indolacetic acid oxidase (106). The resulting increase in indolacetic acid activity may cause an early breakdown of seed dormancy.

### *3.3 Pharmaceutical compounds*

Plants are one of the great resources for pharmaceutical compounds and some of the most important ones, in terms of physiological activity and economic value, are among the S containing secondary metabolites. A significant selection of such compounds has already been presented in this series (89). It may be interesting to note here that none of the classical plant poisons contains S (34). Treatments relying on S containing compounds from plants have a history much longer than the knowledge or identification of the compound itself (109, 122). In alchemy S was one of the three philosophical principles (Sulphur, Merkur and Sal) representing the principle of sun and passion, styling and activity, soul and conciousness, prime potency and love (57). Parts of this history still survive in today's practices of homeopathy and in the rules of biodynamic circles (107, 109). *Achillea millefolium* (common yarrow) for instance is regarded by biodynamic doctrine as the herb with "the highest perfection in utilisation of S" in the plant kingdom (107) but without attributing this compliment to a particular biochemical compound. The best investigated ones among S containing metabolites with pharmaceutical importance are glucosinolates and alliins. Although their mode of action is still not fully understood by modern medicine (31) plants containing alliins and glucosinolates such as garlic and mustard have a long history in popular medicine not only for their general benefits for health and well-being (12, 117, 122) but especially for their action as aphrodisiacs (4, 43, 108, 109, 116). Garlic preparations are regarded highly by health concerned customers and consequently manufacturers of related pharmaceutical products are always searching for the highest alliin concentrations in their raw materials. In the past this has been done mainly by selecting batches by variety and origin. Recently the role of the S supply has been verified as one of the major environmental factors governing the content of these compounds in the plants (see fig. 1 in ref. 50 (this volume)). Therefore a general reduction in the supply of S also reduces the benefits that growers expect from growing these type of crops and this reveals the need for efficient S fertilisation schemes to maintain quality of production.

### *3.4 Baking quality of wheat*

The baking quality of bread-making wheat is a very complex phenomenon which depends on several different factors. One major source of variation is the gluten content of the flour which is closely related to the total protein content of the grain (5). Some very important physical features of the dough, such as its elasticity and resistance to extension, are related to the concentration of sulphur compounds in the gluten fraction (sulphur containing amino acids and glutathione) because they are responsible for the linkages between the protein molecules (5, 17, 29, 32, 55, 59, 114, 121). Thus, the concentration of sulphur containing compounds and also the baking quality of the wheat

grain is not only genetically fixed but also dependent on environmental factors such as sulphur supply (17, 18, 19, 74, 76).

A relationship between sulphur supply and baking quality has been shown by several research groups (17, 19, 74, 76) and outlined as a serious problem for field grown wheat (97, 124). This is confirmed by comparing previously published data for the variety "Avalon" in 1981/82 (19) with data obtained from 1989 samples of the same variety with lower sulphur concentrations (1.72-1.49) and increased N:S ratios (12.7-14.1) in the grains.

The concentration of sulphur in the grain is, however, dependent on the total protein content and both parameters interact to influence the loaf volume (Fig. 10): with increasing protein concentrations in the grain the effect of increasing sulphur concentrations on the loaf volume has a steeper slope and, *vice versa*, at higher sulphur levels an increase in grain protein concentrations results in a greater effect on loaf volume.

A comparison of German and British home-grown wheat varieties with similar characteristics for loaf volume and falling number is given in table 1. The samples were taken in the late 1980s when Germany had reduced its sulphur dioxide outputs, but Britain took approximately 5-8 years longer to reach the same level. The varieties grown at that time in Britain realised the same result in the baking experiment at lower protein concentrations than those grown in Germany. This is the result of the higher sulphur concentration and thus the closer N:S ratio in the British home-grown varieties. It seems as if a lack in protein or sulphur can partly be compensated for by increased concentrations of the other compound (see also Fig. 10). However, increasing S deficiency in German wheat cropping (11) has been compensated for by higher nitrogen inputs, to the detriment of the environment.

Although there are several methods in modern baking technology to improve the baking quality of wheat flour by, for instance, adding L-cysteine during dough preparation (15, 16), millers are still interested in high quality bread-making varieties. Organic growers will have to produce high quality wheat as the possibilities for adding improving agents during processing is limited for "organic" products (104, 110). But with decreasing atmospheric S inputs to agricultural land (87), low baking quality of wheat flour is becoming an increasing problem for organic growers.

### *3.5 Carbohydrate from malting barley and sugar beets*

Carbohydrates derived from malting barley and sugar beets are negatively affected by increasing concentrations of proteins and amino acids in the grain or root, respectively (8, 35, 36, 64). These components tend to accumulate with increasing nitrogen supply and affect crop quality negatively (8, 36). As described in section 2.2.1, S nutrition will only affect this quality factor when it is deficient. Concentrations of N compounds will not respond to S fertilisation in plants sufficiently supplied with S but where S is deficient, there will be an increase in the concentrations of N compounds (7, 44).

### *3.6 Sulphur emissions from biofuels*

The problem of global warming, as a result of steadily increasing $CO_2$ concentrations from the burning of fossil fuels, has highlighted the value of "biofuels" as a new group of agricultural crops. The principle is to ensure short-term re-cycling of $CO_2$ by collecting solar energy via $CO_2$-assimilation in the biomass of plants for use as a fuel, i.e. by burning annual (e.g. wheat) or perennial (e.g. *Miscanthus*) crops which would avoid $CO_2$-enrichments in the atmosphere (24, 30, 103). Power plants for biofuels are most likely to be small, decentralised units in rural areas. To be economically viable, these

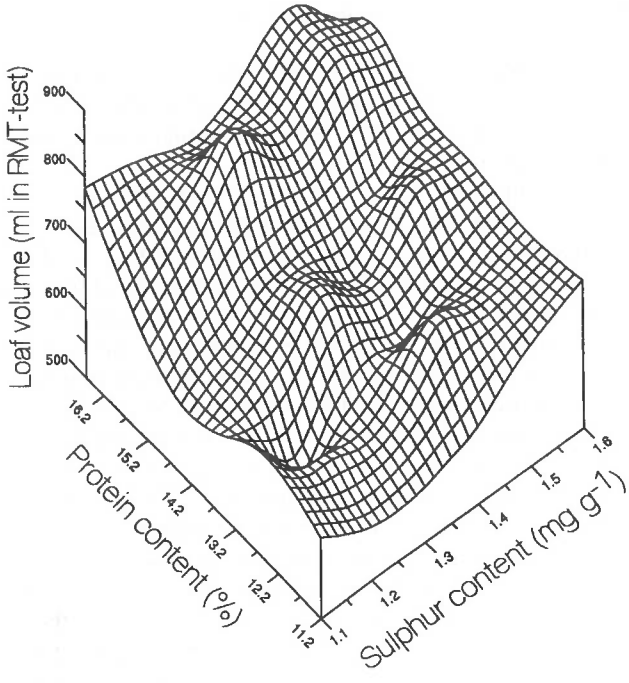


*Fig. 10.* Loaf volume estimated by means of the Rapid Mix Test depending on the protein and total sulphur content of German home grown *Triticum aestivum* (winter wheat) varieties (47).

*Table. 1*: Comparison of quality parameters of German and British home-grown wheat varieties (47)

| Parameter | British "D" / German "B4" | British "B" / German "A6-7" |
|---|---|---|
| Loaf Volume (ml) | 612 / 612 | 717 / 713 |
| Falling Number (secs.) | 215 / 276 | 247 / 381 |
| Protein Content (%) | 10.8 / 13.1 | 12.6 / 14.3 |
| Sulphur Content (mg/g) | 1.38 / 1.25 | 1.46 / 1.35 |
| N:S Ratio | 12.6 / 16.6 | 14.0 / 17.8 |

power plants will require simple but advanced technology which, unlike the power plants used for burning fossil fuels, will probably not have the expensive equipment necessary for removing gases, such as $SO_2$, from the exhaust fumes. Vegetative plant parts have approximately twice the levels of S than the reproductive organs and crops fertilised with S have higher S concentrations in the straw than unfertilised ones (46). As an example, an average harvest of wheat (7 t $ha^{-1}$ grain) will bind 8-10 kg of S per ha in the remaining straw if unfertilised with S, and 26-28 kg if fertilised with S. As more than 50% of the S contained in organic matter is released during thermal combustion (27), biofuels may become a significant source of atmospheric S pollution. This reveals the need for controlled fertiliser regimes to keep the S concentrations as low as necessary for maximum growth (44).

*3.7 Ecological functionality of domesticated plants*

Ecological functionality is defined here as the beneficial contribution of crops to ecosystems. As far as S is concerned, three examples will be presented here: the contribution of crops to the degradation of surface ozone, non point nitrogen losses from agriculture and the function of oilseed rape as forage for honey bees.

Surface ozone concentrations have increased in rural areas over the last decade on average by 1.8 g $m^{-3}$ $y^{-1}$ (Fig. 11). At the same time S concentrations have been declining at a constant rate of 0.45 mg $y^{-1}$ (Fig. 11). Assuming that: a) $H_2S$ emissions from plants decline together with S supply (21, 77) linearly at a rate of 0.57 nmol $m^{-2}$ $h^{-1}$ (calculated from data presented in ref. 94); b) crops have an average leaf area index of 1; c) crops assimilate and reduce S on an average of 100 days a year and 10 h a day; and d) $H_2S$ degrades $O_3$ in a 1:1 ratio; then up to 75% of the observed increase in surface ozone could be attributed to the decrease in the total amounts of S turn-over in the "green part" of the ecosystem. The figures given here are only an estimate and may change depending on the factors considered, but they still outline the important function of S assimilation and reduction in the ecosystem. Despite the importance of this for air quality, the higher sulphur inputs in the past century enabled plants to adapt to increasing environmental stress caused by increasing surface ozone concentrations and, *vice versa,* the decline of the S supply within only one decade (87, 88) may have serious consequences for the stability of recent ecosystems. For example, sulphur deficiency is thought to be one of the reasons why 50% of all forests are damaged. Although sulphur emissions have been cut down drastically over the past 10 years (111), the effect is thought to be due to the combination of reduced resistance (due to sulphur deficiency) and, at the same time, increased environmental stress (118, 123).

Nitrogen losses to the environment. Via the metabolism of amino acids, the utilisation of nitrogen and sulphur are dependent on each another, which means that for the efficient use of high nitrogen levels in agriculture, a sufficient sulphur supply is required. Therefore, increased ecological problems from agricultural crop production are expected because the utilization of fertiliser nitrogen is diminished in sulphur deficient crops (96). This may result in increased nitrogen losses into the environment especially by the leaching of nitrate into the hydrosphere or gaseous losses to the atmosphere. On average, each kg of sulphur unavailable to satisfy the plant's demand, leaves 15 kg of nitrogen with the potential to be lost to the environment. For northern European oilseed rape crops, the actual annual rate of fertiliser nitrogen loss to the environment due to a declining crop sulphur status is estimated to be between 4000 and 6000 metric tonnes (88).

Forage for honey bees. One of the most fascinating symptoms of nutrient deficiency is the "white flowering" of S deficient oilseed rape plants (95). This symptom is often overlooked in the field, because human eyes need at least 10-15 minutes to adapt in order to recognise the white flowers in a flowering rape field. The trigger for the colour change is most likely the increasing sugar concentrations in the tissue (section 2.2.1) due to disorders in protein metabolism. By pigment formation, plants prevent excessive accumulations of free sugars. Thus, white flowering in S deficient crops will most likely occur during periods of high photosynthetic activity. For the phenomenon to occur, two mechanisms could be involved: one major pigment causing the yellow colour of rapeseed flowers is the flavonol, quercetagetin, and its isorhamnetin 3-glycoside (14, 52). Glycosylation of flavonols, however, has a hypsochromic effect which might shift the absorption spectra to the UV range which is invisible for human eyes. The second hypothesis is that the increasing sugar concentrations promote the formation of anthocyanines which would occur in the form of colourless leucoanthocyanines. Like any

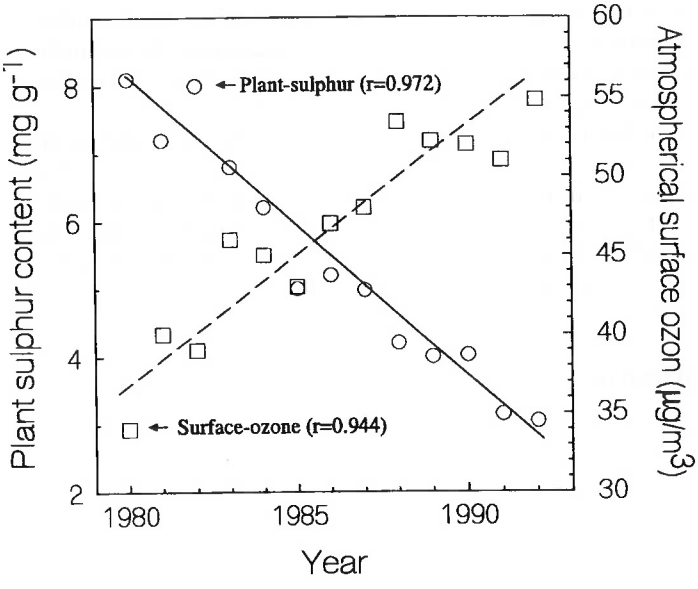


*Fig. 11.* Atmospherical surface ozone concentrations and total sulphur content in younger, fully developed leaves of field grown *Brassica napus* varieties in northern Germany from 1980-1992 (88).

other S deficient tissue of oilseed rape, white petals show lower cysteine, γ-glutamyl cysteine, glutathione and ascorbate concentrations but increased peroxidase activity (98).

Depending on the duration of S deficiency, not only the colour, but also the size and shape of oilseed rape flowers are affected. Reduced S supply for a short time is the reason for white but normally shaped petals. This phenomenon is typical of sites where S deficiency due to decreasing environmental S inputs is just beginning to develop. In regions with an established low S input, such as in all northern European growing areas, S deficient white rapeseed flowers are significantly smaller, with more oval shaped petals (95). The bright yellow colour of flowering fields of rape is attractive to honey bees and, oilseed rape is by far the most important agricultural crop providing forage for honey bees. Crops visited by bees show earlier petal fall probably because they set flowers earlier, resulting in more uniform pod ripening and ease of harvest. This may, therefore, result in higher yields (119). Nectar, however, is the bee's source of carbohydrate and their hovering is the one of the most energy expensive forms of flight. For a "cold" take off the bee requires vast amounts of energy. Compared to civil aviation, this is equivalent to the fuel load a jumbo-jet would use for a whole transatlantic flight. In order to maintain operations it is vital for the bee to refuel on each flower visited and to avoid visiting flowers where the nectar reservoir has already been depleted by another bee. The reflective pattern of flowers provides visitors with clues as to the age of the flowers and presence of food rewards (58), but bees are also influenced by the shape, outline form, and outline length of flowers (67). During senescence of rapeseed flowers, which begins immediately after pollination, the yellow petal colour vanishes and the petals shrink quickly before falling to the ground. A pollinated and fading rapeseed flower is therefore similar to an unpollinated S deficient one. Consequently, the visual message the bee receives from a S deficient rapeseed flower is that of a previously visited flower with an empty nectary. Barth (2) reported that bees prefer yellow flowers to white ones and con-

sequently in S deficient fields much lower bee activity is observed than in S sufficient crops which are bright yellow. Who could have imagined in the beginning of the 80s that the honourable idea of reducing the $SO_2$ emissions from burning fossil fuels (105) would have an impact on honey production twenty years later?

In terms of pollination, this may be of minor interest in common rapeseed varieties which are highly self pollinating. However, the introduction of composite rapeseed hybrids where female and male plants need to grow side by side is much more dependent on pollination by bees. First observations of field grown composite hybrids show increased problems with pollination of hybrids in low sulphur environments. This problem can be attributed to the processes discussed above.

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# LIFE-HISTORY SYNDROMES AND THE ECOLOGY OF PLANTS FROM HIGH SULPHUR-HABITATS

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## Abstract

The reaction of plants to sulphur supply in the natural environment is compared with Grime's life-history syndromes. For the three primary syndromes, i.e. competitor, ruderal and stress-tolerator, characteristic sulphur levels or sulphur compounds were not found.

The adaptation of plants to high-sulphur environments is reviewed for the type of the environment. The avoidance strategy is widespread for gaseous sulphur compounds, derived from volcanoes and lignite burns. In terrestrial and aquatic environments real tolerance to increased sulphur supply is present. The compartmentation of sulphur as sulphate in vacuoles seems to be an evolutionary stable strategy. In marine and coastal environments DMSP is more osmolyte than a detoxification compound, but it plays an important role after volatilization to DMS in the global S cycle.

## Introduction

Plants as part of the biosphere can only exist by their capability to integrate sources from the three abiotic spheres, i.e. the atmosphere, the hydrosphere, and the pedosphere. Whereas most chemical elements necessary for plant growth are extracted from the pedosphere (terrestrial plants) or hydrosphere (aquatic plants) or both (semiterrestrial plants), sulphur together with carbon and nitrogen can be derived from all three spheres due to their different valence states, i.e. chemical speciation. Sulphur is available from very reduced forms such as $H_2S$, $CS_2$, COS and DMS up to the very highly oxidized sulphate ($SO_4$) (cf. Ernst 1993). Although sulphur comes fifth or sixth in quantity of macronutrients (Cram 1990), the attention paid to its impact on the ecological performance of higher plants is low compared to the enormous attention given to carbon dioxide and nitrogen. The chemical state of sulphur in plants can range from strongly reduced sulphur in sulphhydryl to the highest oxidation state as sulphate (Ernst 1990).

Demands for or accumulation of certain amounts of a chemical element incl. nutrient by plants, species and genotypes, have been used to classify plants biogeochemically (Duvigneaud and Denaeyer-De Smet 1968), physiologically (Kinzel 1972) or ecologically (Grime 1979). In this context I will emphasize that each classification will be biased by the evolutionary potentials and restrictions of the various taxonomic categories, in the case of sulphur the high sulphur demand of many cruciferous species in relation to glucosinolate synthesis and the relatively low demand of grasses. In this overview it will be elaborated how sulphur demand and sulphur metabolism is associated with the sulphur supply of various environments. The various reaction patterns will be compared with the above-mentioned classifications with special emphasis on the three life-history syndromes defined by Grime (1979) and related to sulphur-enriched environments.

*Sulphur Metabolism in Higher Plants, pp. 131-146*
*Edited by W.J. Cram et al.*

## Classification of plants in relation to sulphur

Duvigneaud and Denaeyer-De Smet (1968) have based their biogeochemical classification on the concentration of an element in plant parts, mostly leaves. Sulphur-accumulating species have been catagorized as poly- and mesothiophores, those simultaneously rich in salts as halothiophores, species poor in sulphur and other elements as oligophores (Table 1). The thiophores are mostly restricted to saline and gypsum soils, both being discussed later.

Kinzel (1972) based his physiological classification on the concentration of mineral elements and organic acids in the cell sap (Fig. 1). Sulphur accumulates in the vacuole mostly as sulphate and in exceptional situations as phytochelatins (Ortiz et al. 1992) and glucosinolates (Schnug 1990). Plants with high sulphate concentrations in the vacuole belong to the sulphate type, in most cases identical with the sulphate-halophytes sensu Walter (1968) and the halothiophores sensu Duvigneaud and Denaeyer-De Smet (1968).

Grime's (1979) ecological classification is based on physical impacts on and chemical concentration in the various environments. He has suggested that the intensity of disturbance and the intensity of stress in an environment have guided the evolution of life-history strategies. His consideration of life history of plants has resulted in the identification of three major syndromes of life-history traits: competitors, ruderals, and stress-tolerators. By a combination of traits four secondary life-history syndromes are elaborated, all visualised in a triangle (Fig. 2). Grime's classification will be discussed in more detail. It will be shown that the metabolic role of sulphur has enabled plant species to participate in the evolution of all three syndromes, but that the specific characters of "ruderals" (R) and "competitors" (C) combined with the environmental chemistry of sulphur-enriched habitats has mostly restricted plants to the syndrome of "stress tolerator" (S), summarized as the C-R-S strategies[1].

*Table 1.* Biogeochemical classification of plants in relation to their sulphur content (after Duvigneaud and Denayer-De Smet 1968)

| Type | Plant species | Element content (% dry matter) Sulphur | Sodium |
|---|---|---|---|
| Polythiophore | *Gypsophila hispanica* | 4.9 ± 0.7 | 0.05 ± 0.01 |
| | *Ononis tridentata* | 6.3 ± 1.9 | 0.04 ± 0.01 |
| Mesothiophore | *Lepidium subulatum* | 2.8 | 0.02 |
| | *Matthiola tristis* | 1.4 | 0.03 |
| Halothiophore | *Suaeda splendens* | 5.2 | 13.0 |
| | *Plantago maritima* | 1.8 | 11.7 |
| Oligophore | *Ammophila arenaria* | 0.37 | 0.24 |
| | *Festuca rubra* | 0.32 | 0.54 |

[1] To avoid ambiguous meaning of S-abbreviation from "Sulphur" and from "Stress-tolerator" all S further on in this contribution is sulphur.

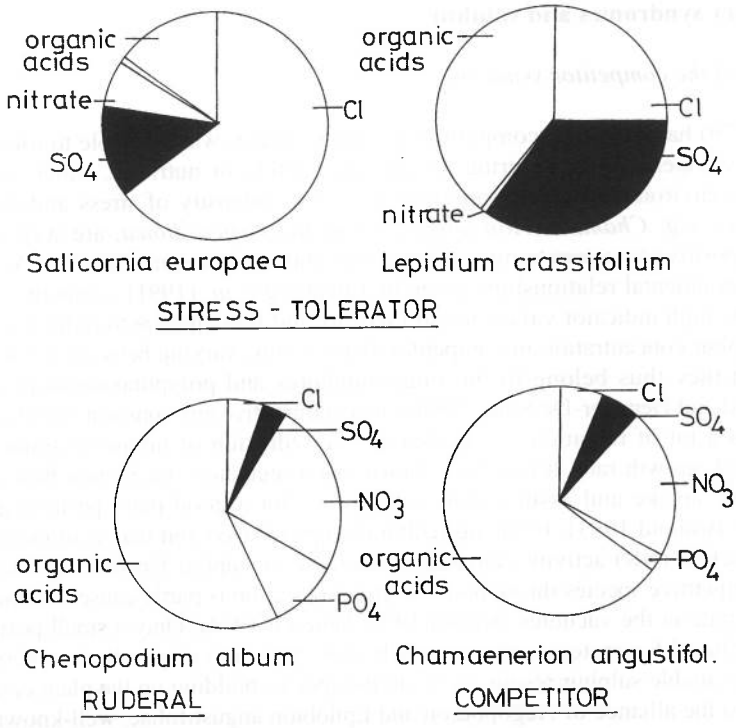


*Fig. 1.* The relative contribution of sulphate, chloride, nitrate, phosphate and organic acids in the vacuoles of leaf cells. *Salicornia europaea* (halophore sensu Duvigneaud and Denaeyer-De Smet; chloride physiotype sensu Kinzel; stress tolerator sensu Grime), *Lepidium crassifolium* (thiohalophore; sulphate halophyte; stress tolerator), *Chenopodium album* (potassionitrophore; oxalate physiotype; ruderal), *Chamaenerion angustifolium* (potassionitrophore; nitrate-malate physiotype; competitor). Data were derived from Albert and Popp (1977), Baumeister and Ernst (1978), Ernst (unpubl.), and Lew (1974).

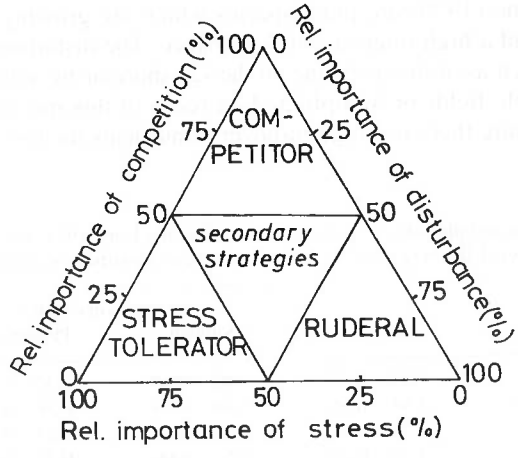


*Fig. 2.* The classification of plant species as competitor, ruderal and stress-tolerator, based on the relative impact of competition, disturbance and various kinds of stress on the life-history strategies (after Grime 1979).

## Life-history syndromes and sulphur

### *Sulphur and the competitor syndrome*

Grime (1979) has defined a competitor as a plant species which is able to tolerate a high degree of competition by ensuring an efficient capture of nutrients, water, light and/or space. The environment of a competitor has a low intensity of stress and disturbance. Competitors, e.g. *Chamaenerion angustifolium* and *Urtica dioica*, are well known for their very positive response to nitrogen and phosphorus (Rorison 1969; Van Andel 1976). In the environmental relationships given by Ellenberg *et al.* (1991) competitors are characterized by high indicator values for soil fertility and for exposure to radiation (Table 2).

The sulphur concentration in competitor plants is low, varying between 0.2 to 0.5% dry matter and they thus belong to the oligothiophores and polypotassonitrophores sensu Duvigneaud and Denayer-De Smet (1968). In a competitive environment rich in nitrate and phosphorus a lot of radiation is required for the reduction of nitrate to aminogroups to ensure a high growth rate. It has been shown that a regulatory interaction between nitrate and sulphate uptake and assimilation is necessary for a good plant performance (for a review, see Brunold 1993). In the agricultural crops tobacco and maize an increase of the nitrate reductase (NR) activity demands an increase in sulphur fertilisation. The low S/N ratio in competitive species throughout the growing season is partly caused by the accumulation of nitrate in the vacuoles (Kinzel 1972, Janiesch 1973). Only a small portion of the nitrate is reduced for protein synthesis. Such a low S/N ratio may indicate a very efficient use of the available sulphur resources by all the species building up the plant communities belonging to the alliance of Aegopodion and Epilobion angustifoliae, well-known for their high nitrate demand and response. On the other hand, a low S/N ratio may be an indication of a limited sulphur supply in competitive environments; which interpretation is substantiated by the lack of S-demanding cruciferous species in such environments. Unfortunately, up to now competitors were not experimentally grown under conditions of simultaneously increasing supplies of nitrate and sulphate, which may unravel their sulphur efficiency.

### *Sulphur and the ruderal syndrome*

Ruderals sensu Grime (1979) are plant species which are growing on sites with a low intensity of stress and a high intensity of disturbance. The disturbance can be caused by natural processes such as at the drift-line on the sea shore or by anthropogenic processes as ploughing of arable fields or trampling. As a result of this mechanical impact on soil and vegetation structure there is a high turnover of nutrients inclusive sulphur. Plant spe-

*Table 2.* Nitrogen, sulphur and phosphorus content (Baumeister and Ernst 1978; Ernst, unpubl.), light (L) and fertility (N) indicator values (Ellenberg et al. 1991) of plant species classified as competitors by Grime (1979)

| Plant species | Content (% dry matter) | | | L | N |
|---|---|---|---|---|---|
| | Nitrogen | Sulphur | Phosphorus | | |
| *Atropa bella-donna* | 5.23 ± 0.72 | 0.42 ± 0.08 | 0.32 ± 0.04 | 6 | 8 |
| *Chamaerion angustifolium* | 4.84 ± 0.68 | 0.46 ± 0.14 | 0.56 ± 0.08 | 8 | 8 |
| *Petasites hybridus* | 3.50 ± 0.12 | 1.15 ± 0.18 | 0.25 ± 0.01 | 7 | 8 |
| *Senecio fuchsii* | 4.29 ± 0.20 | 0.51 ± 0.06 | 0.31 ± 0.02 | 7 | 8 |
| *Senecio sylvaticus* | 5.01 ± 1.04 | 0.64 ± 0.20 | 0.67 ± 0.07 | 8 | 8 |
| *Urtica dioica* | 4.98 ± 0.30 | 0.80 ± 0.17 | 0.42 ± 0.05 | x | 8 |

cies growing at such sites belong to vegetation classes of the Cakiletea maritimae, the Secalinetea, the Chenopodietea and the Plantaginetea majoris. All plants categorized as ruderals are typical indicators of high soil fertility and high radiation, thus conforming to Ellenberg's evaluation (Table 3).

In plant species from the drift-line the S/N ratio is high for instance in the cruciferous *Cakile maritima* (0.38), and low (0.09) in other plant taxa (Denaeyer et al. 1968). Part of the sulphur in *Cakile maritima* and other *Cakile* species is present as glucosinolates (Rodman 1980) which contribute a lot to the high sulphur content of cruciferous species in this and other environments. The sulphur content of glucosinolate-synthesizing species is roughly a factor of two higher than that of other taxa (Denaeyer-De Smet 1970). The occurrence of some cruciferous species in drift-line vegetation and in arable fields, and the moderate accumulation of sulphate in vacuoles of ruderals (Kinzel 1982) are indications of a higher sulphur and nitrogen supply at these sites.

Trampling as a disturbance factor can induce a change of chemical speciation by depriving the soil from oxygen due to soil compaction, reducing nitrate and sulphate to their reduced analogues. Therefore trampling-ruderals may not only suffer from physical disturbance, but also from chemical stress of anoxia, sulphides, and a surplus of plant-available iron and manganese. In terms of the definition of ruderals the latter type belongs to the secondary life history syndrome, i.e. Ruderal Stress-tolerator.

### *Sulphur and the stress-tolerator syndrome*

Stress has been defined by Grime (1979) as "the external constraints which limits the rate of dry matter production of all or part of the vegetation". The constraints may be of a physical nature as a poor penetration of radiation to the forest floor or of a chemical nature. Both types are relevant in the context of sulphur nutrition.

Physical constraints are typical for forests with a closed tree canopy as that of broad-leaved beech forests in Europe. Due to the light dependence of nitrate and sulphate reduction insufficient radiation will hamper photosynthesis and protein synthesis. Plant species at the forest floor will have low light indicator values (L1-L3) sensu Ellenberg et al. (1991). Consequently, in forests with a well developed soil profile at neutral and basic pH-values a surplus of nitrate and sulphate will result in an increase of the concentration in the plant during the growing season (Fig. 3). Although the sulphate accumulation varies among plant species, those forming a closed canopy layer as the grass *Melica uniflora* and the herbs *Corydalis cava* and *Mercurialis perennis* suffer more from self-shadowing than species with an open canopy structure. Therefore the latter group has a smaller sulphate increase during the growing season than the former one. The low radiation on the broad-leaved forest floor restricts the occurrence of cruciferous species in a similar way as that of nitrogen-fixing *Rhizobium*-legume symbioses. Only S-demanding *Allium* species are obvi-

*Table 3.* Nitrogen, sulphur and phosphorus content (Denaeyer et al. 1968), light (L) and fertility (N) indicator values (Ellenberg et al. 1991) of plant species from a drift line classified as ruderals by Grime (1979)

| Plant species | Content (% dry matter) Nitrogen | Sulphur | Phosphorus | L | N |
|---|---|---|---|---|---|
| *Cakile maritima* | 4.5 | 1.70 | 0.38 | 9 | 8 |
| *Elymus farctus* | 3.9 | 0.46 | 0.33 | 9 | 7 |
| *Salsola kali* | 3.4 | 0.29 | 0.28 | 9 | 8 |

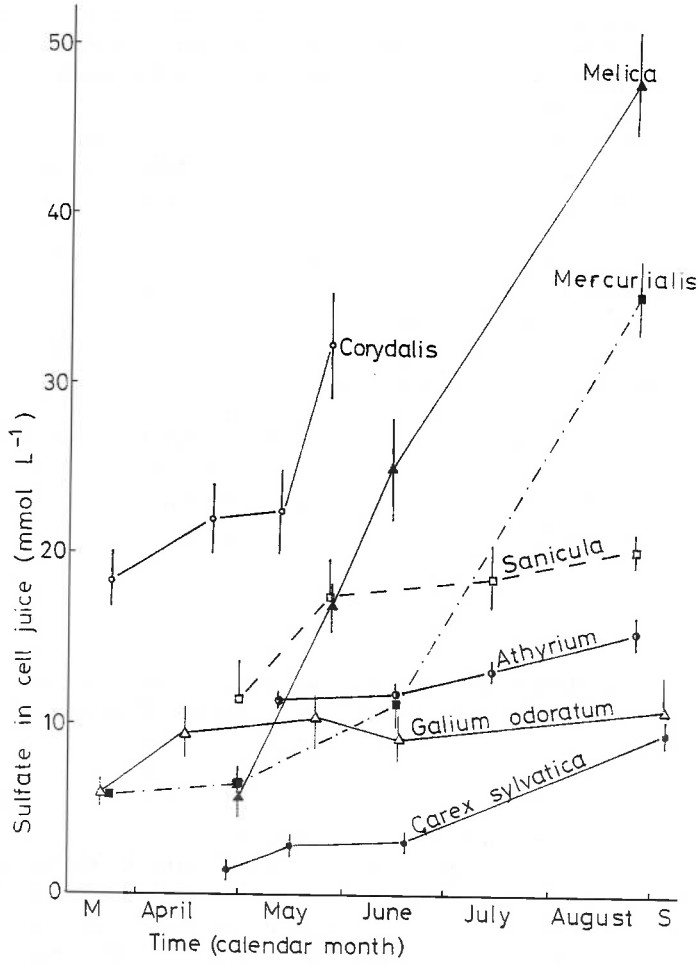


*Fig. 3.* Changes of sulphate concentration in cell juice of several plants in the herb layer from a shadowed beech forest in northern Germany during the growing season (Ernst, unpubl.).

ously able to cope with the low radiation level. They can regulate the sulphur metabolism by partly releasing sulphur as volatile thiopropanol-S-oxides, and by accumulating thiosulphates in the bulb as alliin and ajoene (Sendl and Wagner 1990). Physiologically, accumulation of sulphate in leaf vacuoles may be part of a detoxification mechanism. Ecologically it is more feasible to interpret it as an excellent strategy of sulphur conservation in environments with a low to medium sulphur supply. The low turnover rate of leaf vacuolar S (Bell et al. 1995) allows the build up during the growing season of a huge above-ground S-pool which only in autumn returns to the forest floor for remineralization.

Chemical constraints can be numerous in the various environments. Habitats with nutrient deficiency will stimulate the evolution of low soil-fertility tolerance; plant species growing at these sites will have low fertility values sensu Ellenberg et al. (1991). Habitats with a surplus of essential and non-essential elements, i.e. super-enriched environments, will let evolve plant species or genotypes of wide-spread species which can cope with low soil fertility and simultanuously with a high availability of other elements.

The low biomass production of such multiple-stressed plants may demand other detoxification mechanisms than storage in the vacuole alone. Habitats rich in sulphur and poor in other major nutrients range from those with reduced sulphur (saline marshes) up to those with highly oxidized sulphur (gypsum soils, solfataras). As a consequence plants have evolved various metabolic routes to cope with sulphur excess and to canalize sulphur speciation to such chemical forms in the cell as do not hamper normal plant performance (Ernst 1990). The various pathways and their ecological consequences will be highlighted for plants from such high-sulphur environments.

## Adaptation of plants to high-sulphur environments

Due to the various chemical forms of sulphur S-sources in S-rich environment can be supplied by the atmosphere, the pedosphere and the hydrosphere, and by the biosphere as an intermediate for the fluxes between the three abiotic spheres (Kelley and Smith 1990). Dependent on the form of sulphur, the concentration of the various sulphur species and the exposure time, plant species have evolved different metabolic routes to cope with sulphur excess by avoidance or tolerance.

*Sulphur-enrichment of the atmosphere*

Several sulphur species are volatile: $H_2S$ amd $SO_2$ from abiotic processes, $H_2S$, COS, $CS_2$, and DMS from biotic ones. Enrichment of the atmosphere with $SO_2$ and $H_2S$ is caused by natural processes like the activity of volcanoes, the burning of lignites and peats and natural incineration of forests, and by anthropogenic activities; the negative and positive impacts of the latter activities on plants have recently been reviewed (Ernst 1993). Therefore I will only consider the S-enrichment of the atmosphere by natural processes.

*Volcanoes and S/$CO_2$ vents*

Sulphur dioxide emission from volcanoes adds around 13 Mt $yr^{-1}$ to the Earth's atmosphere (Bluth et al. 1993). The degassing may occur at relatively constant rates as those of Mount Etna in Sicily (Allard et al. 1991), or sporadically at extreme rates like Stromboli volcano in Italy, which varies from 20 to 1800 t $SO_2$ $d^{-1}$ (Allard et al. 1994) and the Nevado del Ruiz volcano in Columbia (Williams et al. 1986). In contrast to sporadic $SO_2$-exposure, a constant rate of emission may be a favourable condition for the evolution of $SO_2$-resistant plants, as long as the metabolic detoxification rate can cope with the emitted concentration. Despite the enormous emphasis on research on anthropogenically caused $SO_2$-injury (Ernst 1993), the investigation of plants in naturally $SO_2$-enriched environments is very limited.

The bareness around the vents of the Kilauea Volcano in Hawaii, emitting about 400 t $SO_2$ $d^{-1}$, indicates that the evolutionary potential for sulphur dioxide resistance is above this amount, which is equivalent to the daily $SO_2$-emission summed over all emission sources in the Netherlands in 1994 (Zonneveld 1995), but concentrated on an essentially smaller area. In contrast, the emission of 3 t $SO_2$ $d^{-1}$ by the Pauahi Crater in Hawaii, being equivalent to nearly 100 ppm, has allowed the evolution of $SO_2$-resistant genotypes in the tree *Metrosideros collina* (Winner and Mooney, 1985). This resistance is based on an avoidance mechanism because resistant plants close the stomata during periods of $SO_2$- exposure whereas sensitive plants leave them open. The recently evolved $SO_2$-resistance of *Geranium carolinianum* at anthropogenically caused $SO_2$-enrichment is a real tolerance. It is related to an increased capacity of the mesophyll tissue to assimilate and detoxify the surplus sulphur (Taylor et al. 1986). Another example of $SO_2$-

resistance of plants in the vicinity of volcanoes is known from *Pinus pumila* in Japan and Kamchatka (Yadav and Bitvinskas 1991). Transplants of dwarfpine genotypes to the $SO_2$-polluted environments of Sudbury in Canada in the early 70s were able to survive in contrast to all other planted coniferous species.

Despite the long-lasting exposure of plants near volcanoes it is surprising that the evolution of $SO_2$-resistance at these sites is very limited. The limited evolutionary potential may be related to the co-occurrence of other environmental extremes, i.e. high temperature and disturbance. High temperature at the fumaroles at Pantelleria in Italy restricted the root growth of *Plantago bellardi* to the surface-near soil (Merola 1959), thus endangering the supply of water and mineral nutrients to the plant during dry spells. Near volcanoes with heavy eruptions, meter-thick layers of volcanic ash and pumice often destroy the vegetation in such a time sequence that the time for evolution of resistance to the atmospheric constraints is too short. The lack of vegetation in those environments fits well with the prediction made by Grime (1979) that high intensity of stress and disturbance will result in bare soil.

The increased amounts of $CO_2$ released by volcanoes and vents together with sulphur (Williams et al. 1986; Allard et al. 1991; Miglietta *et al.* 1993) may improve plant growth in such a way that it may be easier to cope with the excess of volatile sulphur. Enhanced sulphur emission from vents did not affect the sulphur metabolism of indigenous oaks and experimentally exposed crops around S/$CO_2$-vents at Rapolana (Miglietta *et al.* 1993, Tognetti *et al.* 1996).

*Lignite burns and fires*

Incineration of organic deposits and lightning are other sources of sulphur enrichment of the local atmosphere. Bituminous shales along the sea-cliffs on Cape Bathurst, Canada, are burning by spontaneous ignition and have been emitting sulphur dioxide for decades (Hutchinson et al. 1978). Despite sulphur dioxide concentration of about 155 µg S $m^{-3}$ the grass *Arctagrostis latifolia* and the herb *Artemisia tilesii* are able to survive in this naturally air-polluted environment, obviously due to $SO_2$-resistance. An experimental confirmation, however, is not available.

Lightning is the main ignition agent for fires affecting various kinds of ecosystems (peats, heathlands, savannas, forests) in many parts of the world (Kozlowski and Ahlgren 1974, Gill et al. 1981). These fires not only destroy the vegetation, but in areas with a high fire frequency plants are already selected with seeds with a fire-dependent stimulation of their germination. During these fires the sulphur dioxide concentration may be high, thus selecting surviving species for $SO_2$-resistance. This aspect has not achieved any scientific consideration despite the large areas of prescribed burning for management of ecosystems.

*Sulphur enrichment in the pedosphere*

A range of geological and climatological processes can cause an enrichment of rocks and soils with sulphur, either in the reduced or oxidized form. In many sulphur-enriched environments the counter cation will be responsible for the co-selection process in plant species.

*Gypsum soils*

In gypsum soils ($CaSO_4$. 2 $H_2O$) the counter cation to sulphate is calcium. The vegetation of these soils is characterized by plant species with moderate to high sulphur concentration, ranging from 0.20% S in *Ononis natrix* to 6.39% S in *Launaea angustifolia* and 7.4% S in *Ononis tridentata*. The S/N ratio of these plants varies from 0.09 in the

leaves of the shrub *Traganum nudatum* (Chenopodiaceae) up to 7.22 in the leaves of the perennial herb *Erodium glaucophyllum* (Geraniaceae) (Boukhris and Lossaint 1972, 1975; Duvigneaud and Denaeyer-De Smet 1973; Weinert and Sakri 1977; Heinze et al. 1982). Most of the sulphur is present as sulphate; it is feasable to suggest that sulphate is compartmented in vacuoles. Due to the extraction technique used it cannot be decided if the sulphate is bound to flavonoids (Barron et al. 1988) or even precipitated as gypsum. In the latter case it will be completely immobilized with the metabolic consequence that the plant has to allocate other compounds to the vacuole for the establishment of an adequate osmotic pressure (Albert 1984).

In experiments with increasing concentration of sulphate and calcium, Boukhris and Lossaint (1971) have shown that the regulatory potential of sulphur accumulation is very species specific. *Erodium glaucophyllum* lets the sulphur concentration increase linearly with the external supply. The calcium concentration, however, only partly follows this trend. But it is already so high (2.5 mmol Ca $g^{-1}$) at low sulphur supply that all sulphate can be precipitated as gypsum. Other investigated plant species keep the sulphur content nearly constant despite increased external supply, indicating either a limited uptake potential or a very well designed feed-back mechanism.

*Saline soils*

Saline environments are characterized by enhanced concentration of sodium, chloride, magnesium, calcium and sulphur. The duration of inundation determines the redox potential of the soils and the chemical forms of sulphur, ranging from sulphide to sulphate. Plants permanently growing in reduced soil conditions can counteract a low redox potential in the rhizosphere by radial oxygen losses (Armstrong 1982), as long as the oxygen supply to the shoots is not hampered by long-lasting flooding of the shoot. Nevertheless, plant species from wetlands can tolerate up to 50 mM sulphate, but only 1 mM sulfide (Van Diggelen 1988).

One of these daily flooded saline environments is the lower salt marsh. A comparison of three typical plant species of this ecosystem, i.e. *Salicornia europaea*, *Spartina anglica* and *Limonium vulgare*, indicates that at nearly comparable total sulphur concentration, the sulphur form and the proportion of the various chemical sulphur species in the total sulphur content is very plant species-specific (Table 4). The dominant sulphur species in all halophytes is sulphate, being obviously compartmented in vacuoles. Part of this sulphate may be bound to flavones as flavone sulphate (Barron et al. 1988), perhaps up to 50% of the sulphate fraction (Nissen and Benson 1964). Another part of the sulphate can occur as choline-O-sulphate, which is believed to be a compatible solute (Hanson and Gage 1991) with very low concentration in most halophytes (< 1 μmol $g^{-1}$ dry wt.) except

*Table 4.* Sulphur forms in halophytes from lower salt marshes as percentage of dry matter. Data of cholin-O-sulphate are from Hanson and Gage (1991), analysed in plants from the Basin d'Arcachon (France); data for all other sulphur forms are from halophytes in SW-Netherlands (Van Diggelen et al. 1986; Ernst, unpubl.)

| Forms of sulphur in: | *Salicornia europaea* | *Spartina anglica* | *Limonium vulgare* |
|---|---|---|---|
| total sulphur | 0.91 ± 0.10 | 0.87 ± 0.17 | 1.51 ± 0.21 |
| sulphate-S | 0.80 ± 0.07 | 0.25 ± 0.03 | 0.88 ± 0.16 |
| cholin-O-sulphate-S | 0.03 | 0.03 | 0.42 |
| flavone-sulphate-S | not detected | present | present |
| dimethylsulphonio-propionate-S | absent | 0.22 ± 0.06 | absent |

in plants belonging to the Plumbaginaceae. They can contain between 100 and 200 µmol cholin-O-sulphate $g^{-1}$ dry wt.. The corollary of a function of an osmolyte is compartmentation in the cytosol. Another mechanism of resistance to mineral elements is their removal from the cell. Several halophytes can eliminate mineral elements by salt glands which can be quite efficient (Ernst 1974, Rozema et al. 1981, Van Diggelen 1988). The proportion of sulphur in the excreted salts, however, is small. The very efficient salt glands of *Spartina anglica* excrete only 41 nmol sulphate $cm^{-2}$ $day^{-1}$ which is a factor of 15 to 25 lower than the chloride excretion (Van Diggelen 1988). Certain plant species prefer saline soils with sulphate more than those with chloride and occur especially at continental saline sites. Examples are *Reaumuria negevensis* and *Salsola tetrandra* from the Negev (Winter et al. 1976), *Tamarix* species in Europe and Asia, *Lepidium crassifolium* at Lake Neusiedl (Austria, Albert and Popp 1977). These so-called sulphate-halophytes (Walter 1968) or halothiophores (Duvigneaud and Denaeyer-De Smet 1968) are good sulphate accumulators, but the precise chemistry of the sulphate complex is not known.

In contrast to the oxidized forms of sulphur in the plants, some halophytic genera have obviously independently evolved the synthesis of another organic sulphur compound for S-detoxification, viz. dimethylsulphoniopropionate (DMSP). It occurs in the grasses of the genus *Spartina* (Van Diggelen et al. 1986, Dacey et al. 1987) and *Saccharum* (Paquet et al. 1994) and the herbs *Zostera marina* (Potamogetonaceae), a seagrass of the permanently flooded salt marsh (White 1982), and *Wollastonia* (=*Wedelia*) *biflora* (Asteraceae) (Hanson et al. 1994). DMSP can make up 10 to 25% of the total leaf sulphur budget (Van Diggelen et al. 1986, 1987). External supply of sulphide resulted in higher DMSP concentration than with external sulphate supply. In contrast to increasing sulphate concentration with greater external supply, DMSP does not increase its proportion in the total sulphur budget of *Spartina anglica* (Fig. 4). A concentration of 50 mmol sulphate, however, is a factor of five higher than in the salt marsh environment, and may exceed the plant's regulatory capacity. Therefore it has been speculated that in saline environments DMSP is a substitute for cytoplasmic osmolyte glycinebetaine as long as the environment is poor in nitrogen (Colmer *et al.* 1996). Due to the wide-spread occurrence of this compound in marine algae and its role as precursor for the volatile dimethyl sulphide (DMS), DMSP will be discussed in more detail for the marine environment.

The cellular toxicity of sulphide at relatively low concentration demands specific detoxification mechanisms. At the lowest part of European salt marshes *Salicornia europaea* and *Spartina anglica* can survive long-term exposure to 100 µmol $S^{2-}$ $L^{-1}$, whereas the relative growth rate of plant species from the upper-marsh, e.g. *Festuca rubra*, is diminished by 50% (Havill et al. 1985, Van Diggelen et al. 1987). With sulphide exposure flooding-resistant *Aster tripolium* from the lower salt marsh can concert sulphide to sulphate to the same extent as with sulphate exposure (Pearson and Havill 1988). The content of soluble sulphhydryl-S was negligible; the type of organic S, high in roots under sulphide exposure, is unknown. The different reaction pattern to sulphate and sulfide between sensitive and resistant plants can be partially explained by the extent of the root aerenchyma and the related radial oxygen losses, which are high in plants of the lower-marsh soil as a result of selection of resistance to water-logging.

*Sulphur-enriched heavy metal soils*

Other cations in high concentration co-occur with high sulphur contents of soils in many combinations. Due to the type of mineralization, heavy metals can appear in combination with reduced sulphur as in pyrites or with oxidised sulphur as sulphates. The selection pattern of plant species, however, is governed more by the cation than by sulphur-anion. By comparing genotypes of *Thlaspi coerulescens* from soils enriched with copper, zinc,

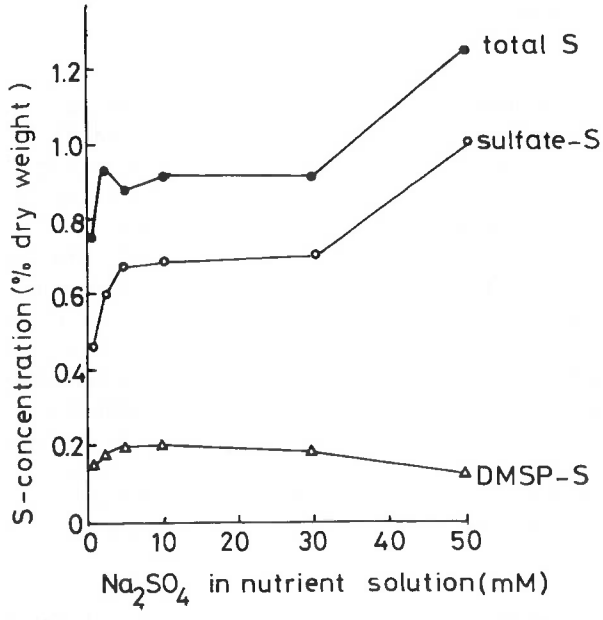


*Fig. 4.* Changes in sulphur content and sulphur speciation in leaves of *Spartina anglica*, grown in a nutrient solution with increased amounts of sodium sulphate (after Van Diggelen et al. 1986).

and cadmium, it was found that there is no difference in metal tolerance when the anionic counterpart is carbonate, sulphate or sulphide. Metal-sensitive genotypes of *Silene vulgaris* can react upon exposure to raised concentrations of arsenic, cadmium and copper by the synthesis of phytochelatins, sulphur-containing polypeptides (Ernst et al. 1992). They have no better performance on soils with a high sulphur status because tolerance to these heavy metals is achieved by protection of the plasmamembrane and a rapid compartmentation of heavy metals in the vacuole, but not by an increased synthesis of phytochelatins (Schat and Kalff 1992). When the weathered ores remain as sulphide,then a combination of sulphide toxicity and heavy metal toxicity may cause evolutionary problems which are very difficult to solve. Tailings with high sulphide concentration ranging from 14 to 27% (Ogram and Fraser 1978) are very difficult to revegetate. The low success may partly due to the surplus of sulphide, partly due to the high concentrations of heavy metals and aluminium. Such an environment demonstrates the limits of evolution and the necessity of an ecologically based environmental management.

In conclusion, plants on S-rich soils have relatively high sulphur contents and S/N ratios; sulphate is the dominant sulphur species and is accumulated in the vacuole. But in many environments the combination of high sulphur concentration and high cation concentration will govern the evolutionary process, often with a superior selection potential of the cation concentration.

*Sulphur enrichment in the hydrosphere*

*Fumaroles and solfataras*

Crevices near or in volcanoes can emit hot vapour and/or hot water (fumaroles) which may be enriched by sulphur, partially also containing HCl and $HNO_3$ (solfataras). The sulphur, originally $H_2S$, can be oxidized to sulphate and is deposited around these vents and on neighbouring vegetation resulting in a whitish cover. The vegetation of these environments has achieved attention from the early phase of ecology, more than 140 years ago (Zollinger 1855), up to recent times (Loetschert 1969, Katoh 1986, Fahselt 1995). The ecological aspects of mineral and especially sulphur nutrition, however, are still unknown. The reason for these shortcomings can be found in the manner of sulphur deposition. Immediately after contact of the hot sulphur-water with the leaves there is such an intimate contact between the deposition and the leaves that it is very difficult to clean the leaves physically or chemically prior to chemical analysis. Removal of the deposit with strong acids is damaging to the epidermal layer with losses of many other elements (Ernst, unpubl.). Therefore it is better to have no data than unreliable data. In the future EDAX-techniques may be helpful in total sulphur analysis and cellular compartmentation, but they will not provide the knowledge about sulphur speciation in these plants.

*Marine environments*

The world oceans are sinks for all chemical elements transported as dust by wind and as dissolved or particulate forms by rivers from the continents to the sea. In contrast to anoxic waters in many continental wetlands, sulphur in the surface-layers of the world oceans is present as sulphate. Its concentration is high (29 mmol $L^{-1}$), a factor of 30 to 40 higher than in fresh water such as springs and rivers (Appelo and Postma 1993). Due to the relatively constancy of sulphate concentrations over time and place, marine algae and plants have evolved resistance to high levels of sulphate and other chemical elements like sodium, chloride, calcium and magnesium and a high efficiency for macronutrients in short supply like nitrogen and phosphorus. In contrast to terrestrial and semi-terrestrial plants on saline soils, marine algae can establish an osmotic potential which is only slightly higher than the surrounding saline water and regulate their mineral budget in a subtle way. As a reaction to the high sulphate supply and the often limited capacity of sulphate storage in the vacuole, some of these algae synthesize DMSP.

The metabolic pathway from sulphate to DMSP is treated by Hanson (1997). The DMSP level in algae is regulated in a species-specific manner (Matrai and Keller 1994), modified by the amount of radiation (Karsten et al. 1991), the salinity (Kirst 1990) and the development cycle (Keller 1991). Calculated on a dry weight basis DMSP concentration can be up to 600 µmol $g^{-1}$ dry mass which is a factor of three higher than in *Spartina* species (Dacey et al. 1987). In addition to its role as osmolytic compound (Edwards et al. 1988) the synthesis of DMSP may be a detoxification pathway of sulphur in this sulphur-enriched waters.

The outcome of the detoxifying mechanisms is not restricted to the individual, but due to the volatilization of DMS, derived from DMSP, it has strong impacts on sulphur fluxes through ecosystems and on the global sulphur cycle. DMSP is an important precursor of dimethylsulfide (DMS) which is a major source (50%) of biogenic atmospheric sulphur (Charlson et al. 1987) affecting the formation and stability of cloud condensation nuclei (Davison *et al.* 1996, Vogt *et al.* 1996). DMSP could be released by viable cells or leached from dying or dead cells. The precise release pathway is still under debate. Due to the enhanced release after grazing of algae by zooplankton, an interaction of phytoplankton, zooplankton and bacteria may regulate a DMSP-DMS cycling in surface water

of the oceans (Kiene 1991). After emission to the atmosphere DMS undergoes photochemical oxidation via dimethylsulfoxide (DMSO) to sulphur dioxide and sulphate (Yin et al. 1986), thus starting once more the sulphur supply to the various ecosystems.

In conclusion, plants have various options to cope with an excess of sulphur. The comparatively higher toxicity of the same sulphur concentration as sulphide than as sulphate demands additional morphological structures like aerenchyma to enhance sulphide oxidation in the rhizosphere. In all sulphur-enriched habitats sulphate is the dominant sulphur component in plants, obviously due to its safe compartmentation in vacuoles.

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# 3-DIMETHYLSULPHONIOPROPIONATE BIOSYNTHESIS IN HIGHER PLANTS

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## Abstract

3-Dimethylsulphoniopropionate (DMSP) is an effective osmoprotectant accumulated by many marine algae and some flowering plants. It is the major biogenic precursor of atmospheric dimethylsulphide, which plays a key role in the global sulphur cycle. The biosynthesis of DMSP from methionine has been partially elucidated in the flowering plant *Wollastonia biflora* (Asteraceae). Methionine is first methylated to give *S*-methylmethionine, after which loss of the amino and carboxyl groups occurs, yielding 3-dimethylsulphoniopropionaldehyde. This is then oxidized to DMSP. The enzymes catalyzing the first and last steps have been identified and shown to be localized in the cytosol and the chloroplast stroma, respectively.

## Introduction

Many marine algae and certain flowering plants accumulate 3-dimethylsulphoniopropionate (DMSP). This is of both environmental and agricultural interest. The environmental interest arises because DMSP is the major biogenic source of atmospheric dimethyl sulphide (DMS), which has a central role in the global sulphur cycle (1, 34). Biogenic DMS is also implicated in acid precipitation (29) and, as the main source of cloud condensation nuclei over the oceans, may be involved in climate regulation (5, 25). The agricultural interest stems from the effectiveness of DMSP as an osmoprotectant and cryoprotectant (20, 41) and from the possibility of enhancing the stress resistance of crops by genetic engineering of the accumulation of such compounds (14, 39). With these interests in mind, we here review the occurrence of DMSP and closely related sulphonium compounds and summarize recent progress in elucidating DMSP biosynthesis in higher plants, particularly that made since our last review (14; see the same volume for reviews of DMSP in algae and of many other aspects of DMSP). Other recent articles have covered various aspects of DMSP catabolism, principally in bacteria and algae (10, 22, 28, 47).

## Occurrence of DMSP and related compounds

The structures of DMSP and three other sulphonium betaines are shown in Figure 1. DMSP is by far the most widely distributed of these compounds. It is accumulated by marine algae from at least six classes: Chlorophyceae, Prymnesiophyceae, Dinophyceae, Bacillariophyceae, Rhodophyceae and Phaeophyceae (3, 21). DMSP is also found in higher plants: high levels (about 5 to 50 μmol $g^{-1}$ fresh weight) have been reported in leaves of *Spartina anglica* (24), *S. alterniflora* (9, 11), *S. foliosa* (30) and *Saccharum* spp. (32) from

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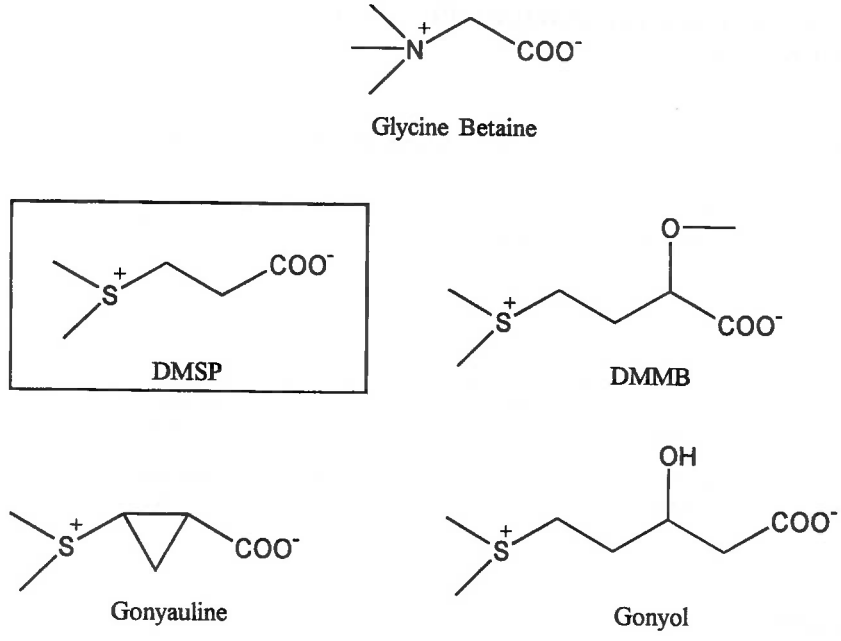


*Fig. 1.* Structures of DMSP and some other naturally occurring tertiary sulphonium compounds. DMMB, 4-dimethylsulphonio-2-methoxybutyrate. Glycine betaine is included to show the analogy between these compounds and betaines, which are quaternary ammonium compounds that contain a carboxylic acid group.

the family Poaceae, and *Wollastonia biflora* (= *Melanthera biflora*) (16, 43) from the family Asteraceae. The *Spartina* species are salt marsh halophytes and *W. biflora* is a salt-tolerant coastal strand plant, whereas *Saccharum* (sugarcane) is a moderately salt-sensitive crop. Other species of Poaceae may have small amounts of DMSP (from 0.01 to around 0.3 μmol $g^{-1}$ fresh weight) (7, 32), as may many dicotyledonous plants (31).

The other sulphonium compounds in Figure 1 have so far only been found in marine algae: 4-dimethylsulphonio-2-methoxybutyrate (DMMB) in various genera of Rhodophyceae (38), and gonyauline and gonyol only in Dinophyceae (27, 36). Another compound, 5-dimethylsulphoniopentanoate, originally reported from flowers of *Diplotaxis tenuifolia* (Brassicaceae) (23), has been shown to be an extraction artifact (15). Lastly, it should be noted that only a few algae and still fewer higher plants have been thoroughly analyzed for DMSP and other sulphonium betaines. Our picture of the distribution of these compounds therefore remains highly incomplete (14, 35).

## Biosynthetic pathway steps

Early radiotracer experiments with the alga *Ulva lactuca* (Chlorophyceae) showed that the carbon skeleton, the S atom and the methyl groups of DMSP are derived from methionine (Met) (13, 19). Subsequent radiolabelling studies with other algae confirmed that Met is the precursor of DMSP (6, 33, 46). While this work established that DMSP originates from Met via methylation, deamination, decarboxylation and oxidation, it did not show the order of these steps or provide information about the intermediates and enzymes involved. Progress in these areas has recently been made with *Wollastonia biflora* (16, 17, 18, 45), as summarized below and in Figure 2.

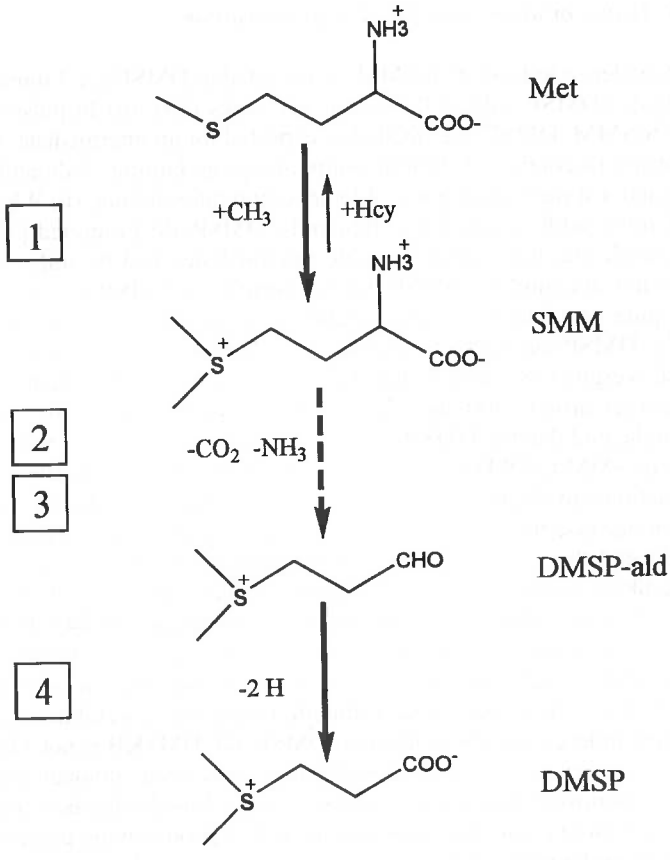


*Fig. 2.* The pathway of DMSP synthesis in *Wollastonia biflora*. The conversion of SMM to DMSP-ald is shown as a dotted arrow as it may involve an intermediate (14,18). The reverse step (thin arrow) appears to be a minor reaction in *W. biflora*. Numbers on the left correspond to the following steps: 1, *S*-methylation; 2, transamination or deamination; 3, decarboxylation; 4, oxidation. Abbreviations: Hcy, homocysteine; Met, methionine; SMM, *S*-methylmethionine; DMSP-ald, 3-dimethylsulphoniopropionaldehyde; DMSP, 3-dimethylsulphoniopropionate.

*Step 1: S-Methylation*

Four lines of evidence from isotope labelling studies with *W. biflora* leaf disks indicate that conversion of Met to *S*-methylmethionine (SMM) is the first step in DMSP synthesis (16). (a) In pulse-chase experiments with [$^{14}$C]Met, SMM had the labelling pattern expected of an intermediate, acquiring label rapidly during the pulse and then losing it during the chase period when [$^{14}$C]Met was replaced with unlabelled Met. (b) [$^{14}$C]SMM was efficiently converted to DMSP. (c) Supplying unlabelled SMM reduced $^{14}$C incorporation into DMSP from [$^{14}$C]Met and caused accumulation of [$^{14}$C]SMM. (d) The dimethylsulphonium group from [$^{13}CH_3$,$C^2H_3$]SMM was incorporated as a unit into DMSP. Detailed studies have not yet been made of other angiosperms. However, leaf disks of sugarcane readily converted [$^{35}$S]Met to [$^{35}$S]SMM, and [$^{35}$S]SMM to [$^{35}$S]DMSP, suggesting that SMM is an intermediate in DMSP synthesis in this species also (31).

*Steps 2 and 3: Trans- or deamination and decarboxylation*

Three lines of evidence indicate that SMM is converted to DMSP via 3-dimethylsulphoniopropionaldehyde (DMSP-ald) in *W. biflora* leaf disks (18). (a) In pulse-chase experiments with [$^{35}$S]SMM, DMSP-ald labelled as expected for an intermediate, whereas three other possible intermediates (3-dimethylsulphoniopropylamine, 3-dimethylsulphoniopropionamide and 4-dimethylsulphonio-2-hydroxybutyrate) did not. (b) When [$^{35}$S]SMM was supplied along with unlabelled compounds, DMSP-ald promoted [$^{35}$S]DMSP-ald accumulation while the three other possible intermediates had no trapping effects. (c) Plants that do not accumulate DMSP did not form [$^{35}$S]DMSP-ald from [$^{35}$S]SMM. DMSP-ald is quite unstable in aqueous solution, and is present only in trace amounts in *W. biflora*. The DMSP-ald content estimated from radiolabelling data was only 0.3-0.6 nmol $g^{-1}$ fresh weight (18), which may be compared with the contents of SMM and DMSP, respectively around 300 and 12,000 nmol $g^{-1}$ fresh weight (16). The radiolabelling data also indicated that the DMSP-ald pool turns over once every 15-30 sec.

Conversion of SMM to DMSP-ald involves loss of the amino and carboxyl groups, but the enzyme reactions involved are not known. It seems improbable that the first reaction is decarboxylation because this would produce 3-dimethylsulphoniopropylamine, and radiotracer data indicated this compound was not an intermediate (see above). There are perhaps two more likely possibilities. One would be a direct conversion of SMM to DMSP-ald, catalyzed by a decarboxylation-dependent transaminase (40,44). This conversion would be unusual inasmuch as there appear to be no other cases in which an amino acid with an α-H undergoes such a reaction. Alternatively, transamination or oxidative deamination could produce the α-keto acid 4-dimethylsulphonio-2-ketobutyrate (DMSKB), which could then undergo decarboxylation to DMSP-ald. DMSKB is not a known natural product and appears never to have been chemically synthesized, although there is indirect evidence that its adenosine analogue (*S*-adenosyl-2-keto-4-methylthiobutyrate) is a natural metabolite in *Escherichia coli* (42). Amino acids with a good leaving group in the γ position are known to undergo ß,γ-elimination upon conversion to the corresponding keto (or imino) acid (8). Since SMM has a good leaving group, $(CH_3)_2S^+$ (37), its α-keto analogue DMSKB would be expected to be unstable and to rapidly decompose, releasing DMS in the process. This does not, however, necessarily preclude DMSKB as an intermediate.

*Step 4: Oxidation*

If DMSP-ald is an intermediate in DMSP biosynthesis, then the last step in the pathway must be the oxidation of the aldehyde group. It was not possible to confirm this directly by supplying [$^{14}$C]DMSP-ald to *W. biflora* leaf disks, due to the chemical instability of DMSP-ald and its rapid catabolism to DMS (18). However, as outlined below, an enzyme catalyzing the conversion of DMSP-ald to DMSP can be readily detected in *W. biflora* leaf protoplast extracts (45).

## Biosynthetic enzymes and their intracellular location

*S-Adenosylmethionine:methionine* S-*methyltransferase (MMT)*

The enzyme catalyzing the *S*-adenosylmethionine (AdoMet) dependent methylation of Met has been purified from *W. biflora* leaves and characterized (17). This enzyme (AdoMet:Met *S*-methyltransferase, EC 2.1.1.12, MMT) is a homotetramer of 115-kDa

subunits; this is unusual inasmuch as methyltransferases are typically monomers or dimers of 20- to 45-kDa subunits (17). Like other methyltransferases that utilize AdoMet (4), MMT is strongly inhibited by *S*-adenosylhomocysteine. It is also inhibited by SMM. SMM and MMT occur very widely in plants (2, 12). MMT is therefore not unique to the DMSP biosynthesis pathway and, as might be expected, antibodies raised against *W. biflora* MMT recognized a 115-kDa polypeptide in partially purified MMT preparations from leaves of various other plants (17).

Apart from conversion to DMSP, SMM has only two known metabolic fates: cleavage to homoserine and DMS, and reaction with homocysteine (Hcy), catalyzed by SMM:Hcy *S*-methyltransferase, to give two molecules of Met (12). The tandem action of MMT and SMM:Hcy *S*-methyltransferase, together with the hydrolysis of *S*-adenosylhomocysteine, can interconvert SMM and Met; this sequence of reactions has been termed the SMM cycle (26). This cycle appears to function only at a low level in *W. biflora* leaves (16).

*DMSP-aldehyde dehydrogenase (DDH)*

A dehydrogenase catalyzing DMSP-ald oxidation to DMSP (DMSP-ald dehydrogenase, DDH) has been detected in extracts of *W. biflora* mesophyll protoplasts, and partially characterized (45). This enzyme has a high affinity for DMSP-ald (apparent $K_m$ = 1.5 μM), which is consistent with the very low levels estimated for DMSP-ald *in vivo* (see above). The enzyme used NAD or NADP as cofactor although NAD was strongly preferred; the apparent $K_m$ for NAD was 6.9 μM vs. 68 μM for NADP. Immunological evidence indicated that DDH is closely related to the last enzyme of glycine betaine biosynthesis, betaine aldehyde dehydrogenase (BADH), which catalyzes a very similar reaction. Thus, anti-BADH serum neutralized DDH activity, and recognized a single polypeptide of the same size as BADH (63 kDa) in immunoblot analyses (45).

*Intracellular location of MMT and DDH*

Following fractionation of *W. biflora* protoplast lysates by differential centrifugation, MMT activity was recovered exclusively from the cytosolic fraction; results from immunohistochemistry were also consistent with a cytosolic location (45). The distribution of DDH was completely different: after differential centrifugation, most of the DDH activity was found in the chloroplast fraction. Analyses of chloroplasts purified using a Percoll gradient confirmed that DDH was mainly ($\geq$ 90%) if not solely chloroplastic and that it was located in the stroma (45).

If the first enzyme of DMSP synthesis (MMT) is cytosolic and the last (DDH) is chloroplastic, then some intermediate of the pathway must move from the cytosol into the chloroplast. Because SMM is chemically stable and DMSP-ald is labile [half-life in physiological conditions approximately 2 min (45)], SMM would seem more suited for inter-compartmental transfer. Consistent with this alternative, intact *W. biflora* chloroplasts converted supplied [$^{35}$S]SMM to [$^{35}$S]DMSP; the conversion was greatest in the light in the presence of $HCO_3^-$ and 3-phosphoglycerate (45).

Taken together, these findings indicate that SMM is made in the cytosol, imported into the chloroplast, and there converted successively to DMSP-ald and DMSP (Figure 3). It follows from this that the yet-to-be identified enzyme(s) catalyzing the SMM $\rightarrow$ DMSP-ald reaction(s) are in the chloroplast. Further, the stimulation of DMSP synthesis by $HCO_3^-$ and 3-phosphoglycerate in the light suggests some link between this reaction and the Calvin cycle.

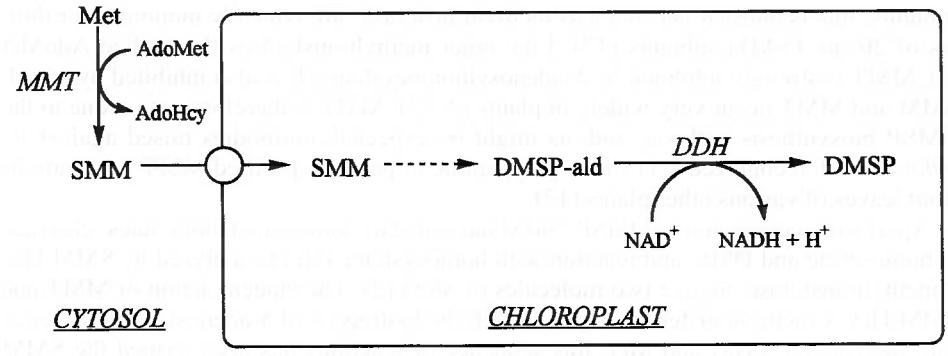


*Fig. 3.* Intracellular compartmentation of DMSP synthesis in *Wollastonia biflora*.

## Acknowledgements

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# A GLOBAL REVIEW OF CROP REQUIREMENTS, SUPPLY, AND ENVIRONMENTAL IMPACT ON NUTRIENT SULPHUR BALANCE

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## Abstract

Agricultural systems worldwide face several critical challenges: in some regions production must be expanded to provide food for growing populations, while in others production levels have to be sustained while striving for the right balance between intensive agriculture and environmental concerns. Sulphur plays a critical role in agriculture as an essential component of balanced fertilization schemes – ensuring optimal efficiency of all nutrient inputs – necessary to maintain higher yields while preserving the environment. The importance of plant nutrient sulphur continues to grow worldwide as food production increases while overall incidental sulphur inputs tend to diminish. Increasing crop production, reduced sulphur dioxide emissions, and shifts in major fertilizer sources have led to worldwide increases of documented sulphur deficiencies. This paper summarizes the main issues regarding plant nutrient sulphur balance in global agriculture.

## Introduction

Over the past decade, agricultural developments worldwide have catapulted sulphur into a critical role for effective agronomic management practices. Differing agricultural, environmental, and political issues and agendas, have resulted in a common crop nutritional disorder worldwide: sulphur deficiency.

Some of the main causes of sulphur deficiency are shared by countries worldwide, including intensive agricultural practices that remove greater amounts of nutrients per unit area and increased use of high-analysis, sulphur-free fertilizers. However, in developed economies, the driving force increasing sulphur deficiencies has been the drastic reduction of atmospheric sulphur dioxide depositions mandated by environmental legislation. On the other hand, in developing regions – particularly in south and east Asia – the leading cause of widespread sulphur deficiency is determined by the intensity of agricultural production needed to feed a growing population. Moreover, food production growth must be sustained on a constant or shrinking amount of arable land. All of these factors combined have led to a dramatic overall decrease in incidental sulphur applications, while sulphur requirements continue to rise.

At current food production and fertilizer utilization trends, The Sulphur Institute projects an annual sulphur fertilizer deficit exceeding 11 million tons worldwide by 2010. Current deficiencies – estimated at about 7.5 million tons – are damaging economically an increasing number of farmers who see the quality and yield of their crops decline. This phenomenon also is stimulating growth in farmers' demand for sulphur-containing fertilizers, which has prompted the industry to research and develop innovative technologies and products to cater to this growing need.

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The diminishing supply of sulphur to arable land has far-reaching implications and consequences. Sulphur plays an important role in the primary and secondary plant metabolism as a component of proteins, glucosinolates, and other compounds that relate to several parameters determining the nutritive quality of crops. Further, recent studies support the positive environmental impact of sulphur in a sound fertilizer management program as it improves nitrogen fertilizer efficiency, thus reducing nitrate leaching into groundwater. This is particularly important considering that fertilizer practices have been increasingly criticized for their role in pollution and environmental degradation. Therefore, the deterioration of the sulphur supply will have several consequences on natural ecosystems that must be considered in future developments for improved soil fertility and environmental quality.

## Regional Plant Nutrient Sulphur Balance

With the continuing reduction in the supply of sulphur from atmospheric emissions in the industrialized world, and the increasing removal of sulphur in higher yields in the developing world, it is not surprising that sulphur deficiency is becoming an increasingly widespread problem. At current food production and fertilizer consumption trends, the Sulphur Institute estimates that the current annual sulphur fertilizer deficit will increase from about 7.5 million tons to over 11 million tons by 2010 worldwide.

Although an overview of the regional plant nutrient sulphur balance in 2010 indicates that the developing economies in Asia – particularly China and India – present the largest long-term deficits, Europe has incurred sweeping sulphur deficiency problems because environmentally mandated sulphur dioxide reductions have occurred so drastically and rapidly (Figure 1). In fact, as shown in Table 1, during the 1980s incidental sulphur applications on European crops decreased dramatically. The sulphur deposited on European fields has fallen from around 50 kg $ha^{-1}$ $yr^{-1}$ in the late 1970s to less than 10 kg today – equivalent to levels measured a century ago (7). The situation in the northern cropping areas of Europe became so severe that Schnug (17) reported sulphur deficiency as the major nutritional problem in arable crops. In the United Kingdom, Sweden, Denmark, Germany, and France sulphur deficiencies also affect wheat and other cereals seriously (16, 17). It is estimated that average yields of rapeseed in northern Europe have fallen by about 5% per year since 1990. Furthermore, surveys conducted by the Agricultural Development and Advisory Service have shown that the area of deficiency in England grew from 24% in 1991 to 39% in 1992 (21). The same report indicates that 100% of rapeseed in Scotland and 40% in England are sulphur-deficient. The latest projections for 2003 indicate that as the government of the United Kingdom is committed to cut sulphur dioxide emissions to 60% of the 1980 level, 23% of the crop land will be at high risk for sulphur deficiency and 27% at medium risk – accounting for virtually all arable areas (2). Remarkably, as clean air legislation has been addressed in central Europe, deficiencies also have been reported recently in wheat and rapeseed cropping areas in the former East Germany and Poland (Schnug, personal communication). These findings confirm that, due to the high mobility of sulphate ions in soils, areas receiving sufficient amounts of sulphur dioxide depositions can become sulphur-deficient soon after emissions are reduced.

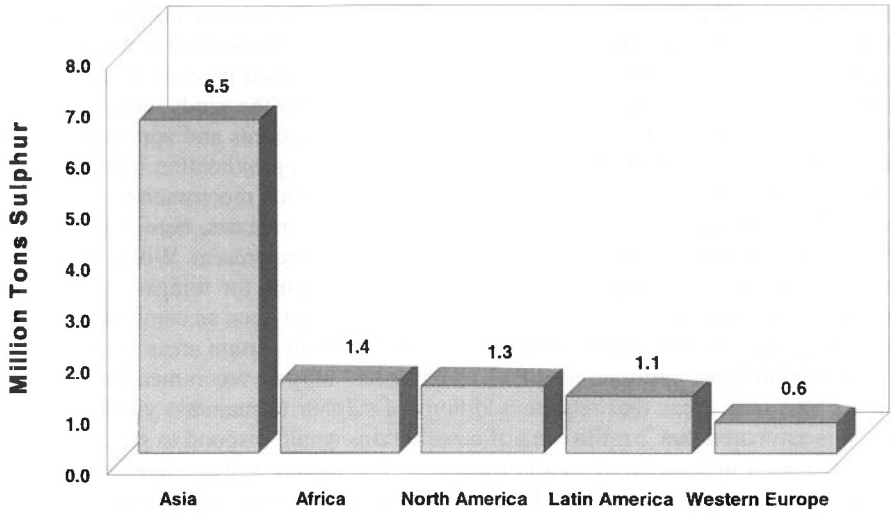


Fig.1. Regional Plant Nutrient Sulphur Deficit Predicted in 2010

*Table 1.* Total sulphur dioxide emissions from anthropogenic sources (Thousand Tons $yr^{-1}$)

| Country | 1980 | 1990 | 1990 per capita (kg $yr^{-1}$) | 1980 to 1990 (% change) |
|---|---|---|---|---|
| Austria | 390 | 98 | 13.1 | -75 |
| Belgium | 828 | 420 | 42.3 | -49 |
| Former Czechoslovakia | 3100 | 2564 | 177.1 | -17 |
| Denmark | 448 | 266 | 52.0 | -41 |
| Finland | 584 | 256 | 51.5 | -56 |
| France | 3338 | 1206 | 21.5 | -64 |
| Germany, Fed. Rep. | 3194 | 1002 | 16.6 | -69 |
| Italy | 3800 | 2406 | 42.0 | -37 |
| The Netherlands | 466 | 238 | 16.1 | -49 |
| Norway | 142 | 60 | 14.2 | -58 |
| Poland | 4100 | 3210 | 83.5 | -22 |
| Spain | 3250 | 2190 | 56.7 | -33 |
| Sweden | 514 | 204 | 24.5 | -60 |
| United Kingdom | 4898 | 3774 | 66.3 | -23 |

Source: UNEP (1993)

With the implementation of the 1990 Clean Air Act Amendments, which mandate an additional 50% reduction (10 million tons) of the 1980 level (21 million tons) by 2000, the United States can be expected to follow a trend similar to that seen in Europe. This is a significant drop considering that the United States' sulphur dioxide emissions decreased from 28 million tons in 1970 to 20 million tons in 1993 – about a 30% reduction. Furthermore, Canada, which has had lower sulphur dioxide emission levels than the United States, is still pushing for stricter emission standards. Between 1980 and 1990, Canada's sulphur dioxide emissions dropped from 4643 tons to 3800 tons – an 18% drop, as compared with 13% in the United States (25). As sulphur emissions continue to decrease, cropping areas will become sulphur-deficient throughout North America.

The last survey of land grant universities conducted by the Sulphur Institute revealed an increase in fertilizer recommendations and rates throughout the United States (8). Adding sulphur to the fertilizer program already has become standard practice in several areas, including much of California, the Pacific Northwest, and for the sandy soils of the Southeast and Corn Belt. Every state has different testing procedures and approaches for formulating sulphur recommendations; however, a general approximation indicates that, in the Mid-Atlantic region, where soils are deficient, sulphur recommendations ranging between 22 to 44 kg $ha^{-1}$ are in place. Among the southern states, between 11 to 22 kg $ha^{-1}$ of sulphur may be recommended where deficiencies are present. Within the southern states, in Louisiana and Arkansas specific recommendations for forages range between 28 to 56 kg $ha^{-1}$. Historically, sulphur deficiencies have not been as common in the Midwest as they have in other parts of the country; however, in certain areas with sandy soils and low sulphur tests, applications of 11 to 28 kg $ha^{-1}$ may be recommended. In Canada, there are vast land areas that require additions of sulphur to maintain yields. In Alberta alone, it is estimated that 2 million ha of cereal grains would respond to sulphur additions while there are 3 million ha of canola (rapeseed) that require sulphur fertilization (14). If current agricultural production and fertilizer use trends continue, the annual sulphur fertilizer deficit in North America is projected to reach 1.3 million tons by 2010 – about 80% of which is projected to occur in the United States.

At present, Asia has to feed nearly 60% of the world's population on approximately 30% of the global land area available for food production (11). Contrary to Europe and North America, there is less environmental pressure regarding sulphur dioxide emissions in Asia. However, the intensification of agricultural production characterized by an increasingly disproportionate nitrogen fertilizer use over the last two decades has created a sulphur nutrient imbalance that is spreading crop deficiencies. Several studies indicate that there is a considerable need for secondary nutrient fertilizers in many regions throughout Asia. In particular, there are large areas of soils categorized as Oxisols and Ultisols in the humid tropics and sub-tropics that are low or deficient in sulphur and other secondary nutrients. To sustain agricultural productivity in these regions, it is becoming increasingly clear that sulphur must form an integral part of fertility programs. Asia has shown an increase in deliberate consumption of sulphur-containing fertilizers since the early 1970s; however, the sulphur requirement is increasing at an even higher rate parallel with food production – about 2% to 3% per year over the past decade. Assuming that current food production and fertilizer consumption trends continue, Asia will have an annual deficit of 4.5 million tons by 2000. This sulphur fertilizer deficit will increase to 6.5 million tons by 2010 unless corrective measures are taken. Within Asia, the developing economies of south and east Asia – particularly China and India – are projected to present the largest long-term potential deficits for sulphur fertilizers. Specifically, China will reach an annual deficit equivalent to 2.6 million tons by 2010; whereas India will reach 1.8 million tons during the same period. Thus, together China and India will represent a deficit equal to 68% of Asia or 39% of the world total.

## Environmental Impact of Sulphur Shortages and Consequences on Agriculture

It is not the intent of this paper to discuss in detail the numerous documented crop responses to sulphur fertilization and the impact of diminishing sulphur supplies on natural ecosystems. However, it is useful to briefly review some of the major implications of sulphur deficiency on agricultural systems.

Several studies have clearly demonstrated a link between sulphur availability and the overall health of a plant. The content of sulphur-containing secondary compounds in plants is not only of importance for nutritive value or flavour, but also for resistance against pests and diseases. For example, sulphur deficiency is responsible for rising light leaf spot levels in the United Kingdom and Germany (18, 26), as well as scab and internal defects in potatoes (13). This has important implications for natural resistance of plants in both agricultural and non-agricultural systems and for alternative agricultural systems where the use of pesticides is prohibited. Here, fertilization is a valuable method to enhance the natural resistance of plants against diseases and insect damage (15). Furthermore, Schnug (19) reported that insufficient sulphur supply may indirectly damage plants by impairing their defense mechanism against damaging oxidizing chemicals such as ozone.

In addition to reducing plant yield, sulphur deficiency also is recognized now as adversely affecting the quality of crops grown for both human and animal consumption. For example, in bread making wheat, the sulphur nutritional status is positively correlated with yield and baking and milling quality (17). A 1992 Home-Grown Cereals Authority (HGCA) survey found there had been a significant decline in the sulphur status of British wheat from the main growing areas that may be responsible for some of the quality problems encountered by end users. Ten years earlier, a similar survey found no evidence of sulphur deficiency. But, in 1992, 7% of the samples had a total sulphur concentration lower than the critical level of 0.12%. A further 34% were in the marginally deficient or 0.12% to 0.14% range. One in ten of the more recent samples had a nitrogen to sulphur ratio greater than 17:1–the level regarded as critical for protein production. In 1982, no tested wheat surpassed this level (1). Furthermore, sulphur fertilization has been shown to influence forage quality through increased vitamin A content of alfalfa, increased chlorophyll content of red clover, increased protein content and amino acid composition of forages, decreased nitrogen to sulphur ratios and non-protein nitrogen and nitrate ($NO_3^-$) levels, and reduced hydrogen cyanide content (24). The $NO_3^-$ concentration in vegetables and forages has become an important criterion for food quality (15). Nitrogen and sulphur are main constituents of proteins; therefore, a shortage in the sulphur supply of crops also affects the utilization of nitrogen within plants for the synthesis of proteins. Thus, sulphur deficiency may cause an enrichment of non-protein nitrogen compounds, including $NO_3^-$, in the plant tissue (10). Therefore, it is important to maintain an optimal sulphur nutritional status to prevent $NO_3^-$ enrichments within plant tissue. The effect of sulphur on forage crop quality also is important because of its ultimate impact on ruminant nutrition and performance. Increased dietary sulphur levels in a number of studies with ruminant animals have shown increased feed uptake, dry-matter digestibility, and improved nitrogen balance, all of which may result in increased meat, milk, and wool production (9). While both sulphur fertilization of forages and direct dietary supplements are beneficial, several studies have shown that dry-matter intake was greater where forage was fertilized with sulphur than where the lower-quality forage diet was supplemented with sulphur (24). This accentuates the importance of supplying the optimal amount of sulphur to agricultural ecosystems, since where a sulphur-deficient condition exists, dietary supplements cannot completely make up for lost production and reduced nutritional efficiency.

Fertilizing practices have been scrutinized as an important factor contributing to pollution, with the contamination of ground water with $NO_3^-$ as one of the most serious problems. Nitrogen and sulphur are both involved in protein biosynthesis; thus, a shortage in the sulphur supply of crops also lowers the utilization of applied fertilizer nitrogen. Besides poor efficiency for nitrogen fertilization, sulphur deficiencies may increase the loss of nitrogen from agricultural soils through volatilization and leaching. Schnug et al. (20) estimated that between 4000 and 6000 tons of nitrogen are lost annually to the envi-

ronment from rapeseed cropping areas in northern Germany, due to insufficient sulphur supply needed to convert nitrogen into biomass. Yet another way for sulphur to benefit the environment by reducing nitrogen leaching to the groundwater is through increased nitrogen fixation. A study in India indicated that increasing rates of sulphur not only increased soybean yield and oil and protein levels in seeds, but also nitrogen fixation from 50 kg nitrogen $ha^{-1}$ without sulphur to 170-175 kg nitrogen $ha^{-1}$ with 40 kg sulphur $ha^{-1}$ (22).

## Economic Implications of Sulphur Shortages

For a fertilizer product to be accepted by farmers, it must fit several criteria, among which economical return for the investment is paramount. Only when the profit margin generated by the extra yield and quality is higher than the cost of application does the application of a fertilizer become attractive to the farmer. Ultimately, the value of sulphur depends on the income from increased yield and the cost of the sulphur applied. Because of the aforementioned extreme sulphur deficiencies throughout Europe, yield benefits are now common on grassland and are increasingly found in arable crops–primarily oilseed rape and wheat. Moreover, sulphur is now showing the kind of responses normally associated with nitrogen.

A series of 32 field trials conducted between 1985 and 1993 in the main grassland areas in the United Kingdom showed an average dry-matter yield increase for sulphur at second-cut of 0.24 tons $ha^{-1}$. On responsive sites, the benefit was 0.71 tons dry-matter $ha^{-1}$. A similar series of 32 trials between 1984 and 1993 on oilseed rape showed an average yield benefit of 0.11 tons seed $ha^{-1}$ overall and 0.34 tons $ha^{-1}$ on responsive sites. From 1992 to 1993, winter wheat field trials funded by the HGCA in the United Kingdom showed grain yield responses averaging 0.5 tons $ha^{-1}$. With a fertilizer cost around US$ 0.40 $kg^{-1}$, the value of sulphur is substantial. The results summarized in Table 2 demonstrate that when applied to deficient crops, sulphur will produce extra yield worth around four- to 15-times the cost of application. Similar remarkable economic returns to sulphur application are shown in Table 3. To assess the value of yield only (without quality considerations), Table 3 details the level of economic response. In all situations, current values suggest only 1% to 2% as the break-even yield response and that average responses will return 10- to 15-times the initial investment (23).

*Table 2.* Value of sulphur as a fertilizer

| Crop | Cost of sulphur (US$ $ha^{-1}$) | Crop Price (US$ $ton^{-1}$) | Value of Extra Yield (US$ $ha^{-1}$) | VCR |
|---|---|---|---|---|
| Second-Cut Silage | | | | |
| Average | 5.90 | 127.00 | 30.00 | 5.10 |
| Responsive Sites | 5.90 | 127.00 | 90.00 | 15.10 |
| Oilseed Rape | | | | |
| Average | 5.90 | 206.00 | 23.00 | 3.80 |
| Responsive Sites | 5.90 | 206.00 | 70.00 | 11.80 |
| Winter Wheat | | | | |
| Responsive Sites | 16.00 | 158.00 | 92.00 | 5.80 |

Sources: Levington Agriculture, Hydro Agri (silage, oilseed rape); ADAS, HGCA (wheat)

*Table 3.* Economics of applying sulphur fertilizers*

| | Grass Silage (cut$^{-1}$) | Winter Rapeseed | Winter Cereals |
|---|---|---|---|
| Yield | | | |
| Increase (tons ha$^{-1}$) | 0.60 | 0.60 | 0.70 |
| Response Value (US$ ha$^{-1}$) | 76.00 | 161.00 | 122.00 |
| Sulphur | | | |
| Application Rate (kg sulphur ha$^{-1}$) | 14.00 | 40.00 | 20.00 |
| Application Cost (US$ ha$^{-1}$) | 5.50 | 16.00 | 8.00 |
| Return | | | |
| Additional Gross Margin (US$ ha$^{-1}$) | 70.00 | 146.00 | 114.00 |
| Economic Response Ratio | 14:1 | 10:1 | 15:1 |

*Assuming average yield responses of 20% for grass silage and oilseed rape, and 10% for winter cereals; fertilizer cost at US$ 0.40 kg$^{-1}$ sulphurS
Source: Kemira Fertilizers

## Developments in Sulphur Fertilizer Technology

The increasing incidence of sulphur deficiencies worldwide has created a growing demand for sulphur fertilizers. To this end, numerous advances have occurred to formulate materials and develop innovative technologies to add sulphur to fertilizers. The main agronomic consideration to be included when developing new sulphur fertilizer products is the fact that plants absorb sulphur mostly in the sulphate form directly from the soil. Therefore, fertilizer sources must supply sulphur directly in the sulphate form (i.e., ammonium sulphate), in a form that can be easily oxidized in the soil into the sulphate form (i.e., micronized elemental sulphur), or in combination (i.e., thiosulphates). Further, sulphate ions are easily leached from soils; whereas, elemental sulphur fertilizers act as slow release sources until oxidation takes place.

Elemental-sulphur-based fertilizers are the most concentrated sulphur carriers; technologies that allow their use in direct applications or as additives to N-P-K fertilizers offer obvious advantages. A considerable amount of trial work has been conducted in Australia and New Zealand using mixtures of elemental sulphur with phosphate rock – in some cases inoculated with sulphur-oxidizing bacteria – and with partially acidulated phosphate rock (6). Additional trial work has focused on methodologies to incorporate elemental sulphur with fertilizers, either during processing or into the finished product (4). Thus, current sulphur fertilizer research in New Zealand is directed towards developing the technology to produce finely divided elemental sulphur to specification and in a form suitable for incorporation into high-analysis phosphate fertilizers or as a degradable granulated product appropriate for dry blending. To accomplish this, an innovative emulsifying process was developed to overcome problems, such as spontaneous ignition, which normally is associated with grinding sulphur (4, 5).

Sulphur bentonite products offer improved agronomic benefits as compared to pure elemental sulphur. The key is to provide the elemental sulphur in an acceptable physical form that can be easily converted to the sulphate form. The finer the particle, the greater the surface area; consequently, the more rapid the oxidation of sulphur to the sulphate form by soil microorganisms. Sulphur bentonite products are produced by a number of processes, whereby molten sulphur is blended with swelling bentonite clays and solidified into usable forms. This material is becoming popular in North America and in Western

Europe because it has a high sulphur concentration to reduce freight and handling costs, allows blending with other fertilizers, resists abrasion, generates little dust, and has an economical and environmentally compatible production process. Commercial sulphur bentonite mixtures were first marketed on a limited basis in the United States in the late 1960s (12). However, earlier products were not successful because they did not possess the right physical and agronomic properties. The principal agronomic consideration is the effect of particle size of the contained sulphur on its availability to plants. Research results generally indicate that particle sizes of 150 mm to 200 mm or smaller are required if elemental sulphur is to be fully effective during the growing season in which it is applied. Recent innovations in production technology and in anti-dusting agents have resulted in the introduction on the market of effective and remarkable products.

Researchers at the University of New England in Australia have taken a new approach for elemental-sulphur-based fertilizers. Adhesion of elemental sulphur to finished products, such as triple superphosphate, diammonium phosphate, and urea, offer new opportunities. This approach represents a deviation from the more established methodology to reform elemental sulphur into granules or prills using bentonite or other binders. To this end, an innovative process (UNE-COAT) has been developed and evaluated (3). This process has shown to solve some problems regarding sulphur fertilizer application in flooded and non-flooded crops and pastures, including improved sulphur dispersion from the granule, and better spatial distribution characteristics. A similar process that is available commercially, has been developed independently by Australia's Western Mining Corporation's fertilizer marketing division, Hi-Fert Pty. Ltd.

Therefore, sulphur can be applied to the soil using a variety of different products (Table 4). Although today the most significant sources are still single superphosphate and ammonium sulphate, new products are increasing their market share. In fact, as farmers' demand for sulphur fertilizers grows, fertilizer producers worldwide are developing innovative products to satisfy the diversified requirements of different agricultural systems.

*Table 4.* Sulphur contributions from various fertilizer sources in 1993-1994 (Thousand Tons)

| Fertilizer source | Sulphur Content | World Use | European Use |
|---|---|---|---|
| Ammonium Sulphate | 24% | 2775.2 | 273.6 |
| Single Superphosphate | 12% | 3615.6 | 92.4 |
| Triple Superphosphate | 1% | 108.1 | 13.6 |
| Ammonium Phosphate | 2% | 348.8 | 0.4 |
| Compound Fertilizers | Variable | 1836.3 | 381.3 |
| Potassium Sulphate | 18% | 253.3 | 39.6 |
| Others | Variable | 57.4 | 30.2 |
| Total Sulphur Consumption | | 8994.7 | 830.7 |

Source: The Sulphur Institute; International Fertilizer Industry Association (IFA)

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# $SO_4^{2-}$ UPTAKE BY MYCORRHIZAL (*LACCARIA LACCATA*) AND NON-MYCORRHIZAL ROOTS OF BEECH (*FAGUS SYLVATICA* L.) TREES.

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## Abstract

The rates of $SO_4^{2-}$ uptake were influenced by external $SO_4^{2-}$ concentrations in a similar way in mycorrhizal and non-mycorrhizal roots of beech trees. However, the responses to S-supply were different for both types of roots. Non-mycorrhizal beech showed diminished/enhanced rates of $SO_4^{2-}$ uptake as a consequence of increasing/decreasing S-supply. In contrast, no such effects were observed for mycorrhizal roots. The thiol and $SO_4^{2-}$ contents of different tissues of the trees were influenced by S-supply and supported the hypothesis of a regulation of $SO_4^{2-}$ uptake by internal S-pools in non-mycorrhizal trees.

$SO_4^{2-}$ is the most important S-compound available to the roots of plants in the soil[1]. In herbaceous plants the uptake of $SO_4^{2-}$ appears to be a well regulated process that is controlled by the plants demand for S[2]. The rates of $SO_4^{2-}$ uptake are also influenced by the supply of other nutrients. High nitrogen supply, for example, leads to increased, low nitrogen supply to diminished rates of $SO_4^{2-}$ uptake[3, 4].

In contrast to herbaceous plants, knowledge of $SO_4^{2-}$ uptake by trees is limited[5]. Due to the long transport distances between different organs and high storage capacities a more complex coordination of transport and uptake processes than in herbaceous plants can be expected. In naturally grown trees both mycorrhizal and non-mycorrhizal roots are present and both are thought to contribute to nutrient uptake[6]. The present study with excised roots of beech trees was performed (a) to characterize $SO_4^{2-}$ uptake in this tree species and (b) to determine the effects of mycorrhization on this process.

The ectomycorrhizal fungus *Laccaria laccata* S238N was cultivated in Erlenmeyer flasks on a modified Marx-Melin-Norkrans-medium containing: 3 g $l^{-1}$ malt extract; 28 mM glucose; 3.7 mM $KH_2PO_4$; 1.9 mM $(NH_4)_2HPO_4$; 0.61 mM $MgSO_4.7H_2O$; 0.43 mM NaCl; 0.34 mM $CaCl_2$; 1.2 ml 1%-$FeCl_3$ and 0.30 µM thiamin HCl (pH 5.6). After a growth period of ca. 8 weeks the fungal biomass was carefully homogenized and aliquots of ca. 100 ml were added to 900 ml of an autoclaved perlite/blond peat mixture. This mixture was incubated at 24°C in the dark. After ca. 8 weeks the fungal mycelium was visible and the substrate was ready for inoculation. For this purpose germinated beech nuts (*Fagus sylvatica* L.) were shelled, surface sterilized and transferred in plastic pots on a substrate consisting of 1 part substrate inoculated as described above and 2 parts sterilized perlite/blond peat mixture. Sterilized substrate without inoculum was used for cultivation of non-mycorrhizal beech trees.

Seedlings were supplied twice a week with 1/5 MS-medium[7], containing 0.35 mM $SO_4^{2-}$. They were grown for 18 to 20 weeks in climate controlled growth chambers (HPS 1500, Heraeus Vötsch, Hanau, Germany) at day and night temperatures of 20° and 15°C,

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and relative humidities of 70% and 80%, respectively. Illumination was provided at 140 to 150 µmol $m^{-2}$ $s^{-1}$ PAR. For preincubation the substrate was carefully removed from the roots and the trees were exposed for 72 h to aerated liquid 1/5 MS media as required for the experiments.

For transport experiments roots were excised and placed in root incubation chambers[4]. The rates of $SO_4^{2-}$ uptake were determined as previously described by Herschbach and Rennenberg[8].

Increasing external $SO_4^{2-}$ concentrations in the uptake compartment of root incubation chambers led to increased rates of $SO_4^{2-}$ uptake by excised roots (Fig.1). Both mycorrhizal and non-mycorrhizal beech trees showed saturation kinetics in the range from 0.01 to 1.0 mM $SO_4^{2-}$. Differences between the types of roots were not observed.

The S-supply during preincubation strongly affected the rates of $SO_4^{2-}$ uptake of non-mycorrhizal, but not of mycorrhizal roots (Fig. 2). In non-mycorrhizal trees S-depletion led to increased rates of $SO_4^{2-}$ uptake, whereas excess S caused significantly lower rates of uptake. However, roots of *L. laccata* inoculated trees showed neither stimulation of $SO_4^{2-}$ uptake in response to S-depletion nor decreasing uptake rates at higher $SO_4^{2-}$ supply.

The contents of the most important soluble S-compounds in the shoot increased with increasing S-supply (Table 1). L-Cysteine and glutathione contents in leaves and bark were slightly lower in non-mycorrhizal than in mycorrhizal trees at S-depletion and normal S-supply. However, the thiol contents in the fine roots were independent of S-supply and mycorrhization. The $SO_4^{2-}$ contents in leaves and bark of non-mycorrhizal trees were generally lower than in mycorrhizal plants especially at S-depletion. The $SO_4^{2-}$ contents of non-mycorrhizal fine roots increased if more $SO_4^{2-}$ was available to the plants, whereas mycorrhizal fine roots seemed to be independent from S-supply.

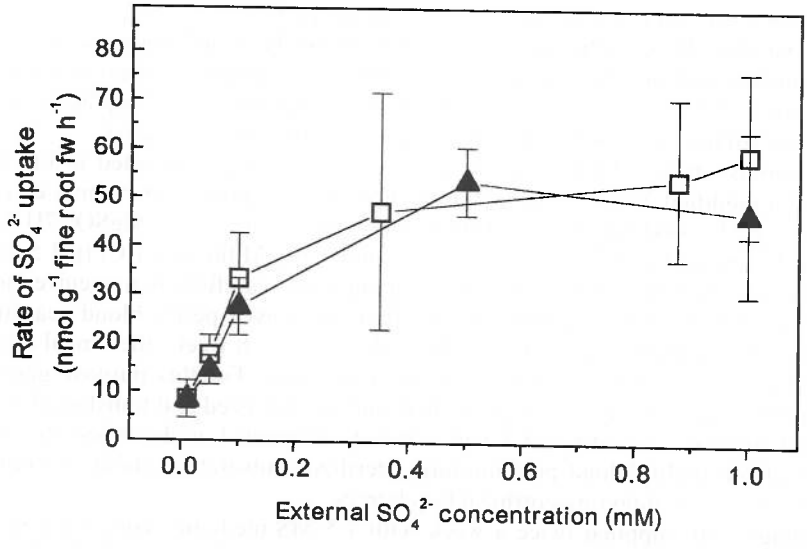


*Fig. 1.* Concentration dependence of rates of $SO_4^{2-}$ uptake. Mycorrhizal (▲) and non-mycorrhizal (□) trees were preincubated for 72 h in liquid 1/5 MS media containing 0.35 mM $SO_4^{2-}$. The roots were excised and placed in root incubation chambers containing 0.01 to 1.0 mM radiolabeled $SO_4^{2-}$ (specific activities ca. 1.5 to 1500 kBq µmol$^{-1}$ $SO_4^{2-}$). After 6 h the incubation was stopped, roots were cut at the borders of the compartments and radioactivity was measured in the different root sections. The rates of $SO_4^{2-}$ uptake were calculated according to Herschbach and Rennenberg[8]. Data are means of 4 to 6 independent experiments with 4 to 6 roots each.

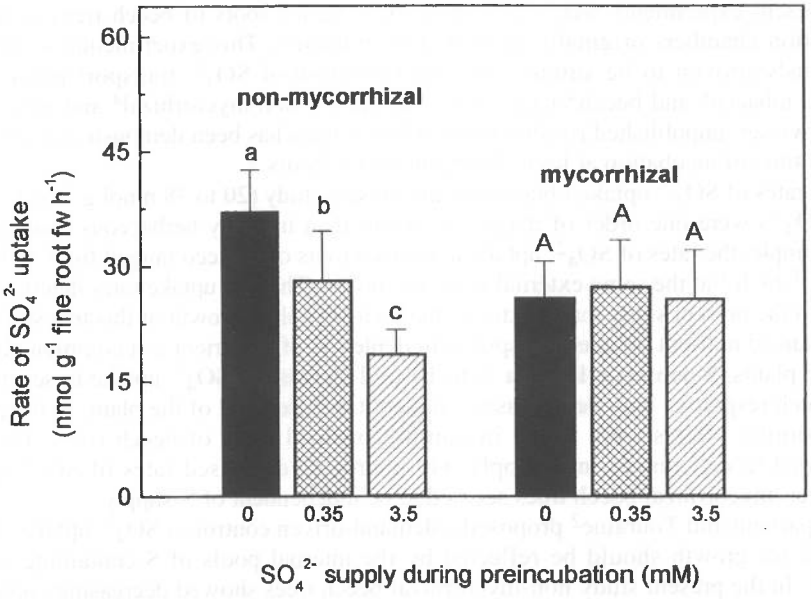


*Fig. 2.* Influence of S-supply on rates of $SO_4^{2-}$ uptake. Mycorrhizal and non-mycorrhizal beech trees were pre-incubated for 72 h in 1/5 MS media lacking S or containing 0.35 mM or 3.5 mM $SO_4^{2-}$. Roots were excised and placed in root incubation chambers containing 100 µM radiolabeled $SO_4^{2-}$ (specific activity ca. 15 MBq $mmol^{-1}$). The incubation was stopped after 6 h. The radioactivity measured inside the roots and the exudation compartment of the chamber was used to calculate $SO_4^{2-}$ uptake[8]. Data are means of 4 to 6 independent experiments with 4 to 6 roots each. For statistical analysis data were subjected to Student's t-Test. Significant differences are indicated by different letters.

*Table 1.* Influence of S-supply on the contents of soluble S-compounds. Mycorrhizal (+) and non-mycorrhizal (-) beech trees were incubated for 72 h in liquid 1/5 MS media lacking S or containing 0.35 mM or 3.5 mM $SO_4^{2-}$. The trees were dissected into the tissues indicated. Thiols were determined by a modification of the protocol of Schupp and Rennenberg[9]. $SO_4^{2-}$ was extracted after homogenization of the tissue in distilled water and was analyzed by ion chromatography (Dionex DX-100, Dionex, Idstein, Germany). Data are means of at least 2 independent experiments with 4 replicates each. Significant differences were determined by ANOVA and are indicated by different letters

| Organ | $SO_4^{2-}$ supply (mM) | $SO_4^{2-}$ content (µmol $g^{-1}$ fw) | | L-cysteine content (nmol $g^{-1}$ fw) mycorrhization | | glutathione content (nmol $g^{-1}$ fw) | |
|---|---|---|---|---|---|---|---|
| | | - | + | - | + | - | + |
| leaves | 0.00 | $1.9 \pm 0.8^{c}$ | $4.1 \pm 1.6^{b}$ | $11 \pm 8^{b}$ | $21 \pm 9^{b}$ | $187 \pm 157^{d}$ | $405 \pm 172^{c}$ |
| | 0.35 | $4.5 \pm 1.6^{b}$ | $4.3 \pm 2.0^{b}$ | $16 \pm 8^{b}$ | $27 \pm 12^{b}$ | $327 \pm 92^{cd}$ | $488 \pm 169^{bc}$ |
| | 3.50 | $7.6 \pm 1.5^{a}$ | $8.0 \pm 5.3^{a}$ | $66 \pm 23^{a}$ | $43 \pm 27^{a}$ | $680 \pm 154^{a}$ | $623 \pm 190^{ab}$ |
| bark | 0.00 | $1.5 \pm 0.9^{c}$ | $2.8 \pm 1.5^{b}$ | $7 \pm 2^{c}$ | $6 \pm 2^{c}$ | $121 \pm 38^{c}$ | $139 \pm 24^{c}$ |
| | 0.35 | $2.3 \pm 1.2^{bc}$ | $2.2 \pm 1.9^{bc}$ | $9 \pm 4^{bc}$ | $10 \pm 5^{bc}$ | $178 \pm 74^{bc}$ | $245 \pm 61^{ab}$ |
| | 3.50 | $3.5 \pm 1.9^{b}$ | $5.6 \pm 2.9^{a}$ | $12 \pm 2^{b}$ | $18 \pm 3^{a}$ | $194 \pm 53^{bc}$ | $292 \pm 102^{a}$ |
| wood | 0.00 | $5.5 \pm 2.1^{b}$ | $14 \pm 4^{a}$ | $4 \pm 2^{c}$ | $6 \pm 2^{abc}$ | $121 \pm 54^{c}$ | $131 \pm 57^{c}$ |
| | 0.35 | $8.2 \pm 5.6^{ab}$ | $8.2 \pm 5.0^{ab}$ | $6 \pm 3^{bc}$ | $7 \pm 3^{ab}$ | $145 \pm 14^{bc}$ | $291 \pm 153^{a}$ |
| | 3.50 | $13.6 \pm 3.2^{a}$ | $8.7 \pm 7.1^{ab}$ | $10 \pm 3^{a}$ | $7 \pm 2^{abc}$ | $301 \pm 85^{a}$ | $274 \pm 78^{ab}$ |
| fine roots | 0.00 | $2.2 \pm 0.9^{c}$ | $5.4 \pm 1.2^{a}$ | $11 \pm 7^{ab}$ | $15 \pm 6^{a}$ | $104 \pm 54^{a}$ | $91 \pm 39^{a}$ |
| | 0.35 | $3.3 \pm 1.4^{b}$ | $4.1 \pm 3.1^{ab}$ | $7 \pm 5^{b}$ | $10 \pm 2^{b}$ | $83 \pm 57^{a}$ | $91 \pm 35^{a}$ |
| | 3.50 | $5.0 \pm 1.5^{a}$ | $4.9 \pm 1.2^{a}$ | $8 \pm 2^{b}$ | $11 \pm 3^{ab}$ | $98 \pm 28^{a}$ | $108 \pm 24^{a}$ |

The present experiments were performed with excised roots of beech trees in the root incubation chambers originally described by Pitman[10]. This experimental system was previously proven to be suitable for determination of $SO_4^{2-}$ transport processes of excised tobacco[8] and beech[4] roots. $SO_4^{2-}$ uptake by non-mycorrhizal[4] and mycorrhizal (Kreuzwieser, unpublished results) roots of beech trees has been demonstrated to be linear with time of incubation at least during the first 6 hours.

The rates of $SO_4^{2-}$ uptake obtained in the present study (20 to 38 nmol $g^{-1}$ fw $h^{-1}$ at 0.1 mM $SO_4^{2-}$) were one order of magnitude lower than in many herbaceous plant species. For example, the rates of $SO_4^{2-}$ uptake in excised roots of tobacco ranged from 450 to 950 nmol $g^{-1}$ fw $h^{-1}$ at the same external concentration[11]. The low uptake rates determined for beech in the present study may be due to the relatively slow growth of this tree species.

Enhanced nutrient uptake in response to depletion of a nutrient is a common phenomenon in plants. S-depletion led to a 3- to 10-fold increase of $SO_4^{2-}$ uptake in several species. Such responses may be necessary to meet the S-demand of the plant. In the present study similar effects were found in non-mycorrhizal roots of beech trees. The roots responded to differences in S-supply with increased/decreased rates of $SO_4^{2-}$ uptake. However, mycorrhizal beech trees seemed to be independent of S-supply.

Lappartient and Touraine[2] proposed a demand-driven control of $SO_4^{2-}$ uptake. This S-demand for growth should be reflected by the internal pools of S-containing components[12]. In the present study non-mycorrhizal beech trees showed decreasing contents of S-compounds in response to S-depletion, accompanied by higher rates of $SO_4^{2-}$ uptake. These findings support the hypothesis of a regulation of $SO_4^{2-}$ uptake by internal S-pools. However, the situation in mycorrhizal trees remains unclear. The fungal symbiont strongly affected S-metabolism of beech trees, since in mycorrhizal trees S-depletion had little or no effect on both the contents of soluble S-compounds and the rates of $SO_4^{2-}$ uptake. It may therefore be assumed that the mycorrhizal fungus provides a "buffer" for short-term fluctuations in S-supply. Further studies are required to test this assumption.

## Acknowledgment

The authors thank Dr. J Garbaye for providing cultures of *L. laccata*. This study was financially supported by the Deutsche Forschungsgemeinschaft (DFG) under contract no. Re515/4.

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# CLONING, EXPRESSION AND PURIFICATION OF FRAGMENTS OF THE PLANT SULPHATE TRANSPORTER

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## Abstract

Two different fragments of the *Stylosathes hamata* high affinity sulphate transporter *shst1* were cloned into prokaryotic expression vectors, pET and pTRB, and amplified in *E. coli*. Successful over-expression of the SHST 1 fragments was achieved with both vectors. The recombinant proteins were enriched and purified to homogeneity either by gel electrophoresis and subsequent electro-elution or by affinity chromatography, and are currently being used for antibody production.

Recently the cloning of genes encoding three different sulphate transporters from the tropical legume *Stylosanthes hamata* cv. Verano has been reported[1]. Two of these genes code for high affinity transporters mediating sulphate uptake by the roots. The third gene encodes a lower affinity transporter, expressed at low levels, throughout the whole plant, and is thought to be involved in intercellular or subcellular transport of sulphate (Smith et al. 1995). These cDNAs for sulphate transporters now offer the tools to tackle many unanswered questions concerning the mechanism of sulphate uptake, the regulation of the expression of the transporters and their intracellular distribution particularly in response to nutrient availability.

The cDNAs of two different genes for *St. hamata* sulphate transporters, *shst1* and *shst3*, were subject to restriction digests to obtain fragments of definite size and sequence (Figure 1). From *shst1*, a high affinity sulphate transporter exclusively expressed in root tissue, three different fragments were produced for cloning: SHST 1.1 representing 536 amino acids, spanning the whole cDNA except for the first 151 amino acids, SHST 1.2, 390 amino acids in length representing the middle section of the gene, and SHST 1.3, a small C-terminal fragment of 144 amino acids. One short fragment was also cloned from the lower affinity transporter *shst3*, expressed in the whole plant at lower level as compared to the high affinity transporters as judged by mRNA abundance: SHST 3.1, representing 146 C-terminal amino acids. According to their restriction sites, the fragments were cloned into different expression vectors: for *shst1.1* and *shst3.1* a pET-28 vector (Novagen) was chosen, providing a sequence coding for 6 consecutive histidine residues (His-tag) at the N- and C-terminus of the recombinant protein, which can be used for affinity purification. *Shst1.2* and *shst1.3* were cloned into pTRB vectors, β-galactosidase fusion vectors, which also allowed easy identification of the recombinant protein, either by determination of β-galactosidase enzyme activity or by molecular mass on SDS-PAGE. The vector/*shst* constructs were transformed into competent *E. coli* host cells, BL 21(DE3) for the pET vectors and BMH 71-18 for the pTRB vectors, by heat shock.

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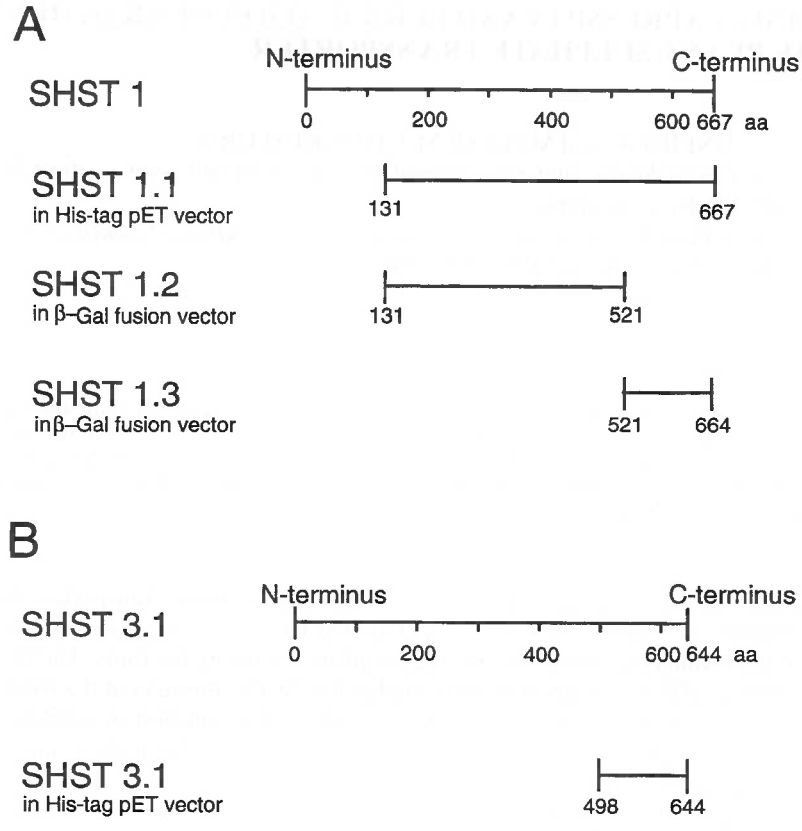


*Fig. 1.* Summary of peptide fragments generated by cloning sections of *Stylosanthes hamata* sulphate transporter genes. The length of the translated cDNAs and the various cloned fragments are given as amino acid residues (aa). A: fragments cloned from *shst1*. B: fragment cloned from *shst3*.

Selection for recombinants took place on growth media containing the appropriate antibiotics, i.e. kanamycin for pET vectors and ampicillin for pTRB vectors. Colonies were tested for the presence of the vector construct by digest with selected restriction enzymes. Only those colonies identified as positive, i.e. containing the constructs, were used for expression studies.

The expression of the recombinant protein was induced by the addition of isopropyl-β-D-thiogalactoglucopyranoside (IPTG, 1mM) to the growth medium. The expressed proteins were identified on SDS-PAGE after lysis of the bacterial cells (Figures 2 and 3). Over-expression of the SHST 1.1 fragment in the pET vector could not be observed in crude cell lysates. With SHST 1.2 and SHST 1.3, however, an over-expression of β-galactosidase fusion proteins was observed (Figure 2). Whereas SHST 1.3 was expressed very strongly (Figure 2, lane 5; 132 kD), the expression of SHST 1.2 was only weak (Figure 2, lane 3; 160 kD). Therefore, SHST 1.3, representing a C-terminal fragment of the transporter protein, was chosen for further purification of the recombinant protein. This was achieved by running the crude lysate on preparative SDS-PAGE and subsequent electro-elution of the recombinant SHST 1.3 β-galactosidase fusion protein from the gel. The electro-eluted protein (Figure 2, lane 6) was used, after concentration, for

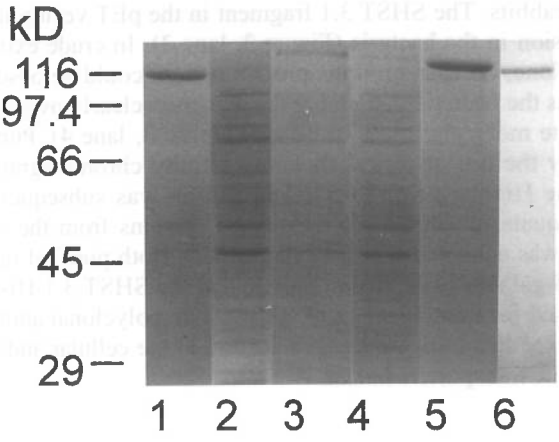


*Fig. 2.* Expression and purification of SHST 1 β-galactosidase fusion proteins. The *E. coli* strain, BMH 71-18, containing the expression vector was induced for expression of the recombinant protein by addition of 1mM isopropyl-β-D-thiogalactopyranoside (IPTG). Expression was carried out at 30°C for 5h. Samples were taken after different time intervals, cells harvested by centrifugation, resuspended in lysis buffer containing SDS and boiled for 15 min. The lysate was separated on a SDS-PAGE. For purification of the recombinant protein, the band containing the fusion was cut off the gel and the protein subsequently electro-eluted. Electro-eluates were washed several times to remove excess SDS and concentrated by ultrafiltration. 1: β-galactosidase subunit, 116 kD; 2: 0h expression of SHST 1.2 β-galactosidase fusion protein; 3: 5h expression of SHST 1.2 β-galactosidase fusion protein; 4: 0h expression of SHST 1.3 β-galactosidase fusion protein; 5: 5h expression of SHST 1.3 β-galactosidase fusion protein; 6: purified electro-eluted SHST 1.3 β-galactosidase fusion protein.

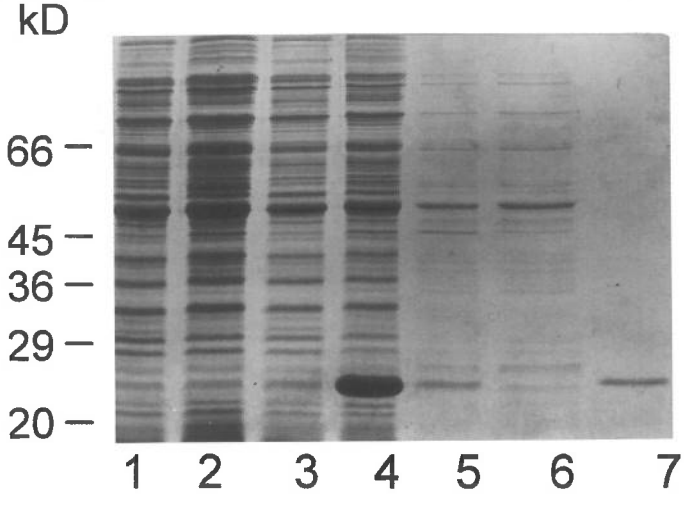


*Fig. 3.* Expression of SHST 3 His-tagged fusion protein. The transformed *E. coli* strain, BL 21(DE3), was induced for expression with 1mM IPTG. Expression was carried out at 30°C for 5h. Cells were harvested and resuspended in lysis buffer. To aid lysis and fragment DNA, short ultrasonic pulses were applied. After centrifugation the supernatant containing the soluble proteins was loaded onto a nickel chelating resin specifically designed for affinity purification of His-tagged proteins. Elution of the bound His-tagged protein was achieved with imidazol buffer. Insert-free pET-28 vector 0h (1) and 5h (2) after induction; SHST 3 recombinant protein 0h (3) and 5h (4) after induction; (5) crude cell lysate as loaded onto the nickel chelating column; (6) protein fraction not binding to the column; (7) purified His-tagged SHST 3 recombinant protein.

the immunisation of rabbits. The SHST 3.1 fragment in the pET vector also showed pronounced over-expression in the bacteria (Figure 3, lane 3). In crude extracts expressing just the pET vector alone, no change in the protein pattern could be observed (Figure 3, lane 1 and 2), whereas the bacteria containing the construct clearly over-expressed a protein of an approximate molecular mass of 20 kD (Figure 3, lane 4). Purification in this case was achieved by the use of nickel chelating affinity chromatography specifically designed to retain the His-tagged proteins. The elution was subsequently carried out under conditions adequate to release the His-tagged proteins from the column. A high grade of purification was achieved (Figure 3, lanes 5-7). Both purified recombinant proteins, the SHST 1.3-β-galactosidase fusion protein and the SHST 3.1-His-tagged protein are currently being used for immunisation of rabbits. The polyclonal antibodies obtained should then provide excellent tools for the localisation of the cellular and subcellular distribution of the sulphate transporters *in situ.*

## Acknowledgement

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# ISOLATION AND CHARACTERIZATION OF A HIGH AFFINITY $SO_4^{2-}$ TRANSPORTER FROM *HORDEUM VULGARE* cv.KLONDIKE

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## Abstract

The cDNA for a high affinity $SO_4^{2-}$ transporter (*hvst1*) of barley was isolated by heterologous expression in *E.coli*. The cDNA was a partial clone which contained 1.2 Kb of the 3' end of the gene. The 5' end of the gene was isolated using RACE-PCR. We have sequenced *hvst1* (genebank accession number U52867), and found it to have high homology with genes encoding high affinity $SO_4^{2-}$ transporters in *Stylosanthes hamata*. Hvst1 expression levels were examined as a function of $SO_4^{2-}$ concentration in growth medium and found to be most highly expressed in media with 10 μM $SO_4^{2-}$. Expression levels were also examined as a function of duration of S-deprivation. Maximum expression (2 fold increase) was evident after two hours and remained high for 48 hours (end of the experiment). Preliminary data suggest the presence of at least two transporters in barley, having a high degree of sequence homology.

The first step of sulphate assimilation is the entry of sulphate into plant cells. Smith *et al.*[1] isolated a $SO_4^{2-}$ transporter from higher plants, using heterologous expression in yeast. By this approach, making use of an yeast $SO_4^{2-}$ transport mutant (*SUL1-*), they isolated three genes, *shst1*, *shst2*, and *shst3*, which encode two high affinity and one low affinity $SO_4^{2-}$ transporters, respectively, in the tropical forest legume *Stylosanthes hamata*.

Heterologous expression has been used to isolate other ion transporters from plants. Examples of these include the high affinity potassium transporter *HKT1* from wheat[2], and the ammonium transporter *AMT1* from *Arabidopsis thaliana*[3].

The present work describes the isolation and characterization of a high affinity $SO_4^{2-}$ transporter from *Hordeum vulgare* cv. Klondike. The gene *hvst1* encoding the $SO_4^{2-}$ transporter was isolated in an *E.coli* expression system.

Growth of barley: Seeds of barley (*Hordeum vulgare* cv. Klondike) were surfaced sterilized using 1% bleach solution for 10 min then rinsed with distilled $H_2O$. The seeds were imbibed overnight and thereafter germinated in sand for 3 d, after which they were transferred to hydroponic tanks containing 1/10 Johnson's solution (100 μM $SO_4^{2-}$). Potassium levels were monitored daily, and tanks were topped up with all other nutrients accordingly. Plants were harvested after 4 d of hydroponic growth. For experiments examining the withdrawal of $SO_4^{2-}$, $Mg(NO_3)_2$ replaced $MgSO_4$.

cDNA Construction: Poly (A)+ RNA was extracted from total RNA (2 h $NO_3^-$ treated roots) using Fasttrack mRNA isolation system (Invitrogen). A cDNA library was constructed using ZAP-cDNA synthesis kit (Stratagene), and a cDNA synthesis for RACE-PCR reactions was constructed using Marathon cDNA synthesis system (Clonetech). RNA was isolated using Trizol reagent (Gibco/BRL).

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Northern Blots: Twenty micrograms of total RNA was separated by 3 h electrophoresis at 6 V $cm^{-1}$ in 1.2% agarose containing 2.2 M formaldehyde. The RNA was transferred to hybond N+ nylon membrane (Amersham) in 20 x SSC, by capillary blotting. RNA was fixed by microwaving for 2 min. The membranes were probed as per manufacturers' instruction (Amersham).

DNA Sequencing: DNA sequencing was determined on Applied Biosystems 373A DNA sequencer, using FS taq, with gene specific primers.

Gene Sequence: We have isolated a truncated gene encoding the high affinity $SO_4^{2-}$ transporter. This gene fragment was approximately 1.2 Kbp in size containing the 3' end of the gene. The 5' end of the gene was isolated using RACE-PCR. The complete gene *hvst1* is 2457 bp in size and encodes a protein of 660 amino acids. The protein sequence reveals twelve membrane spanning regions.

Gene Induction and Expression: It is known that sulphur starvation increases the capacity of $SO_4^{2-}$ uptake[4]. To investigate the response of *hvst1* expression to sulphur deprivation we conducted an experiment in which we withdrew $SO_4^{2-}$ for 2, 6, 12, 24, and 48 h. Roots were harvested, total RNA extracted and probed with 3' fragment of *hvst1*. The expression of *hvst1* in response to S withdrawal was rapid and increased levels of expression were apparent within 1 h of removal of exogenous sulphate. After 2 hours, *hvst1* was maximally expressed, corresponding to a 2-fold increase (figure 1A). High levels of expression continued during the entire experimental period (48h). Resupply of $SO_4^{2-}$ to 2 h S-starved plants, down regulated the expression of *hvst1* (figure 1b). Gene expression was repressed when L-cysteine (100 μM) was present in the nutrient solution during $SO_4^{2-}$ withdrawal (figure 1B).

Steady State Expression: Barley plants were grown at 4 different $SO_4^{2-}$ concentrations *viz.* 10, 50, 100 and 250 μM, which were maintained for 5 days. The highest *hvst1*

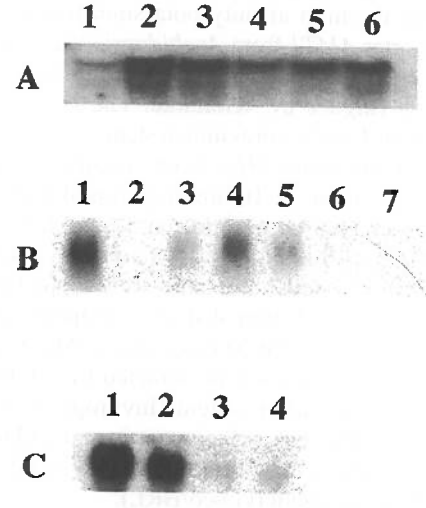


*Figure 1.* Expression of *hvst1* in roots of barley. 1A) Time course of withdrawal of $SO_4^{2-}$. Lane 1, no withdrawal; lane 2, 2h; lane 3, 6h; lane 4, 12h; lane 5, 24h; and lane 6, 48h. 1B) Resupply of S. Lane 1, 2 h S-starved; lane 2, 2h S-starved then $SO_4^{2-}$ resupplied for 2 h; lane 3, resupplied for 1 h; lane 4, resupplied for 30 min; lane 5, resupplied for 15 min; lane 6, 2 h S-starved with 100 μM cysteine; lane 7, 0.1 mM grown plants. 1C) Steady state $SO_4^{2-}$ levels. Lane 1, 10 μM grown plants; lane 2, 50 μM grown plants; lane 3, 100 μM grown plants and lane 4, 250 μM grown plants.

expression was observed in plants grown in 10 μM $SO_4^{2-}$ (figure 1C). Very little expression occurred at 100 and 200 μM $SO_4^{2-}$. The low level of expression was presumably due to the down-regulation of *hvst1* transcription.

The cloning and sequencing of a gene encoding a high affinity $SO_4^{2-}$ transporter in barley adds one more ion transporter system to a growing list of systems for which genes have been identified. This knowledge enables the study of regulation of ion transport, formerly restricted to studies at the whole plant level, to proceed on a substantially more definitive level. Further it enables long-standing controversies regarding the interpretation of kinetic analyses to be put to rest. Details arising from the current work on *hvst1* gene establish an important basis for further analysis of the regulation of sulphate uptake in plants and of the processes involved in adjusting root sulphate uptake to shoot sulphur demand. Detailed knowledge of the sulphate transporters and the feed-back mechanisms involved in sulphate uptake and translocation is essential in order to assess possibilities for enhancing crop sulphur acquisition by overexpression of the gene.

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# PURIFICATION AND CHARACTERIZATION OF APS SULPHOTRANSFERASE FROM SPINACH LEAVES

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## Abstract

A new assay method for APS sulphotransferase (APSSTase) was developed. Using this method, two APSSTase isoenzymes were highly purified from spinach leaves and radish seedlings.

Adenosine 5'-phosphosulphate (APS) sulphotransferase (APSSTase) is thought to be an enzyme which transfers the sulpho-group of APS to a carrier compound with a thiol group in the assimilatory sulphate reduction pathway of higher plants[1]. Currently APS sulphotransferase activity is assayed based on release of $[^{35}S]SO_2$ using [$^{35}$S]APS as a substrate[2]. This method is sensitive but time consuming.

We had the idea to develop an APSSTase assay based on the coupled reaction with sulphite reductase and *O*-acetylserine(thiol)lyase. Sulphite released by the APSSTase reaction in the presence of excess dithiothreitol was converted to cysteine by coupling with yeast sulphite reductase and cabbage *O*-acetylserine(thiol)lyase and the cysteine formed was determined colorimetrically[3]. Yeast sulphite reductase (100-fold) and cabbage *O*-acetylserine(thiol)lyase (100-fold) were partially purified by ammonium sulphate fractionation and column chromatography. APS was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and further purified with HPLC. The reaction mixture for the APS sulphotransferase assay contained 25 μmol potassium phosphate buffer, pH 8.0, 500 μmol $Na_2SO_4$, 50 μmol $Na_2CO_3$, 4 μmol *O*-acetylserine (OAS), 30 μmol DTT, 1 μmol NADPH, 0.2 μmol APS, 0.01 U sulphite reductase, 0.3 U *O*-acetylserine(thiol)lyase, and the enzyme solution in a final volume of 1 ml. After incubation at 30°C for 30 min, the reaction was stopped by addition of 1.5 M trichloroacetic acid. After the centrifugation at 10,000g for 1 min, the supernatant was mixed with the acid ninhydrin reagent[4]. The mixture was then heated for 10 min, then cooled quickly before addition of 2.0 ml ethanol. Cysteine formed was determined by measuring the absorbance at 550 nm. One unit (U) of APSSTase activity was defined as the amount of the enzyme catalyzing formation of 1 mol cysteine per min under the condition. The reaction mixture without APS was used as the blank. APSSTase activity could be detected in leaves of all higher plants tested. The values of the activities obtained here were comparable to those reported so far ($0.43\text{-}1.86 \times 10^{-3}$ unit/g fresh weight).

Although APSSTase had been purified from *Euglena*[5] and *Porphyra yezoensis*[6], the enzyme has been only partially purified from higher plants[1]. We purified APSSTase from spinach leaves using ammonium sulphate fractionation and column chromatography with DEAE cellulose and Phenyl Sepharose. We found two active components after Phenyl Sepharose HP column chromatography (Fig.1). A typical purification is shown in

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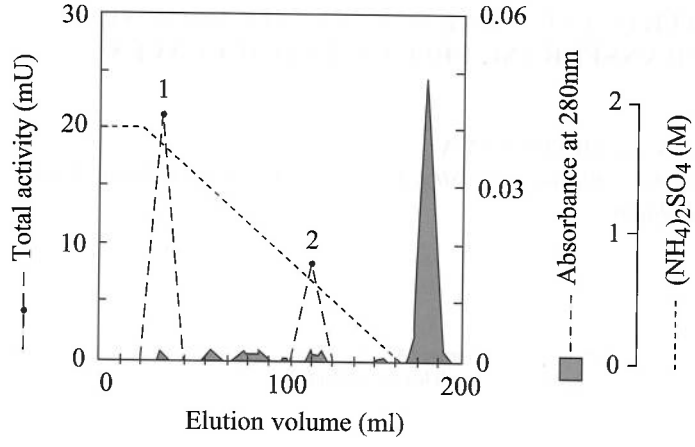


*Fig. 1.* Elution Profile of APSSTase from a Phenyl Sepharose HP Column.
Active fractions of APSSTase after DEAE cellulose column chromatography were applied onto a Phenyl Sepharose column and washed with phoshate buffer, pH 8.0, containing 1 mM EDTA and 10% glycerol (PEG buffer), supplemented with 1.8 M $(NH_4)_2SO_4$. APSSTase was eluted with a linear gradient of 1.8 to 0 M $(NH_4)_2SO_4$ in PEG buffer.

Table 1. One of two fractions, APSSTase 1, was highly purified i.e. 2000 fold. The SDS-PAGE analysis of the APSSTase 1 still showed the presence of some contamination by other proteins. Recently, it was reported that both ATP sulphurylase[7] and *O*-acetylserine(thiol)lyase[8] of higher plants are presented in isozymes, one existing in the plastids and the other in free cytosol. The two active fractions of APSSTase we found may therefore originate from the plastids and free cytosol, respectively. We also highly purified two APSSTases from radish leaves. None of the APSSTases purified utilized PAPS as a substrate.

*Table 1.* Summary of The purification of APSSTase from spinach leaves. Purification of APSSTase was started with 1,800 g of fresh spinach leaves

| Procedure | Total protein (mg) | Total activity (Unit) | Specific activity (Unit/mg protein) | Fold |
|---|---|---|---|---|
| $(NH_4)_2SO_4$ | 1560 | 1330 | 0.79 | 1 |
| DEAE cellulose | 41.4 | 196 | 4.73 | 6 |
| Phenyl Sepharose HP | | | | |
| APSSTase 1 | 0.05 | 84.2 | 1684 | 2130 |
| APSSTase 2 | 0.15 | 30.3 | 202 | 256 |

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# LIGHT AND SULPHUR SOURCES MODULATE mRNA LEVELS OF SEVERAL GENES OF SULPHATE ASSIMILATION

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## Abstract

The regulation of several genes encoding enzymes of sulphate assimilation in *Arabidopsis thaliana* has been investigated with respect to the effects of light and sulphur nutrition. mRNA of the investigated enzymes was demonstrated under all conditions but was predominant in light-exposed tissues. Sulphate deficiency triggered an increase of mRNA contents whereas feeding of cysteine and methionine led to lowered mRNA concentrations.

The assimilation of sulphate represents one of the basic pathways of macronutrient incorporation into cell metabolism. Uptake and reduction of sulphate are co-regulated by photosynthesis, nitrate assimilation and by the demand for reduced sulphur of various cellular reactions[1,2,3]. After uptake of sulphate into the plant cell, the assimilation proceeds first via two ATP-dependent activation steps, that are catalyzed by ATP-sulphurylase and adenosine phosphosulphate kinase (APS-K), respectively, yielding phosphoadenosine phosphosulphate (PAPS). In the free pathway of sulphate reduction, termed after its exclusively unbound intermediates, PAPS is reduced by a thioredoxin-dependent PAPS-reductase and subsequently by a ferredoxin-dependent sulphite reductase (SiR)[4]. The insertion of the resulting free sulphide into cysteine as the first stable organic sulphur compound marks the final step of assimilation. The reaction proceeds from serine via the activated intermediate O-acetylserine and is catalyzed by the cysteine synthase complex which consists of serine acetyltransferase (SAT) and O-acetylserine (thiol) lyase (OAS-TL).

The regulation of sulphate assimilation as determined by physiological and biochemical experiments is assumed to be controlled by the intracellular pools of sulphate, cysteine and possibly O-acetylserine[2,5,6]. An indication that the observed altered extractable activities of sulphate assimilation enzmyes may be caused by gene expression is suggested by an increase of mRNAs encoding OAS-TL[7] and SAT[8] from *Arabidopsis thaliana,* and of a sulphate uptake transporter from *Stylosanthes hamata*[9] in response to short-time sulphate deficiency. In order to begin a molecular analysis of the regulatory network that controls the assimilation of sulphate and the distribution of reduced sulphur, we have previously cloned cDNAs encoding APS-K[10], SiR[11], two isoforms of OAS-TL[7], as well as SAT[8] from *A. thaliana*. These molecular probes for enzymes of sulphate activation, reduction and insertion were applied to study expression of these genes in relation to light, tissue specificity and adaptation to different sulphur nutrition conditions in *A. thaliana* plants.

The expression of APS-K, SiR, OAS-TL, and SAT was measured in mature rosette leaves and roots as well as in etiolated and light-grown seedlings (table 1). The four mRNAs were detectable under all conditions tested but were predominant in light-exposed tissues. SAT mRNA was 5.5-fold more abundant in leaf than in root, but

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*Table 1.* Distribution of sulphate assimilation mRNAs in *A. thaliana*. Radioactively labeled cDNA probes of APS-K, SiR, OAS-TL, and SAT were hybridized in Northern experiments with total RNA isolated from four week old rosette leaves and roots as well as from 10 day old seedlings (including roots) that had been grown etiolated or in a 8 hour light period. 10 µg of each RNA sample was resolved on a 1.3% denaturing agarose gel, blotted on a Nylon membrane and simultaneously hybrized to DNA probes at high stringency[7]. Quantification was achieved by subsequent incubation with a 18S rRNA encoding probe as a reference. Detection from three independent blots was carried out with a PhosphoImager (Fuji BAS 1500) and average values are presented.

| cDNA Probe | Root | Mature leaf | Etiolated seedling | Green seedling |
|---|---|---|---|---|
| APS-kinase | 1 | 2.8 | 1 | 3.9 |
| Sulphite reductase | 1 | 1.5 | 1 | 8.3 |
| O-Acetylserine (thiol) lyase | 1 | 1.2 | 1 | 1.8 |
| Serine acetyl transferase | 1 | 5.5 | 1 | 1.9 |

increased only 1.9-fold upon transfer from dark to light. In contrast, SiR mRNA was only 1.5 times higher in leaves than roots but increased 8.3-fold in response to light. The smallest differences were observed for OAS-TL under those conditions which might indicate additional functions since this enzyme was reported to accept β-substituted alanines as substrates in other plants[12]. The plastid localization of APS-K[10], SiR[11], and OAS-TL[7] seems evident from their primary structure and is largely confirmed by these data. The gene product of SAT was attributed to an organelle[13], however, the data presented here do not allow an unequivocal assignment of this SAT isoform to either plastids or mitochondria. Tissue specific and light dependent expression of the four genes exhibited similar patterns but existing differences in relative abundance of the transcripts remain to be analyzed for their significance at the translational level once antibodies against the proteins are available.

The adaptation of *A. thaliana* to the availability of sulphate was investigated in plants grown on minimal (6 µM) and regular (2.5 mM) sulphate (figure 1). Plants were generally grown under sterile conditions in Gamborg's B5 nutrient medium[18] in order to avoid consumption of sulphur compounds by microorganisms. Intracellular sulphate and glutathione were low in plants from minimal sulphate medium and high in regularly raised plants. The intracellular capacity for sulphate was at least five times higher than for glutathione and apparently was not exausted even at the highest external sulphate concentration tested. In contrast, upper glutathione levels were controlled and reached about 200 nmol (g fresh weight)$^{-1}$ at 0.8 mM sulphate in the medium (data not shown). Cysteine concentrations exhibited only minor differences between minimal and regular sulphate grown plants, indicating a tight control under these conditions as observed for *Lemna*[16] and pea[15].

The mRNA contents of APS-K, SiR, and OAS-TL were 1.5 to 2 times higher under sulphate deficiency than at 2.5 mM sulphate supply. Since cysteine concentrations were similar in both tissues, intracellular sulphate and/or glutathione could have mediated the differences of the sulphate assimilation mRNAs. This was not a general reaction of RNA metabolism, as 18S rRNA levels remained unchanged with and without sulphate.

We now asked whether plants supplied with sufficient reduced sulphur would still express genes of sulphate assimilation. *A. thaliana* plants were grown at sterile B5 medium with minimal and regular sulphate that was supplemented with 2 mM cysteine or 1.5 mM methionine. Both amino acids were able to substitute for sulphate as sulphur source, leading to phenotypically normal growth. External cysteine resulted in intracellular cysteine concentrations of 960 and 750 nmol per g fresh weight in sulphate starved and regular plants, respectively, as determined after complete reduction with DTT[17]. Cysteine

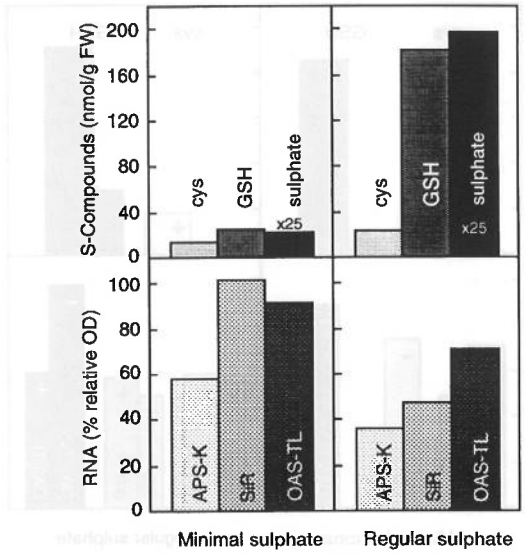


*Fig. 1.* Sulphur metabolites and mRNA contents in *A. thaliana* plants grown on minimal (6 µM) and regular (2.5 mM) sulphate medium. Cysteine (cys) and glutathione (GSH) were determined by monobromobimane derivatisation and HPLC[17], sulphate was quantitated by a modified barium-rhodizonate method[20]. Note that sulphate contents are to be multiplied by factor 25. Hybridization with mRNA of APS-K (light shaded bars), OAS-TL (dark shaded bars), and SiR (black bars) was carried out as described in table 1.

exceeded glutathione contents 2.2-fold regardless of the presence of sulphate in the medium, suggesting that the control of cysteine levels might not apply under these conditions. The contents of APS-K, SiR, and OAS-TL mRNA declined with increasing external cysteine at both minimal and regular sulphate. The decrease was between 20% and 45% for the three genes, which is considered relatively small compared to the high internal cysteine.

In plants methionine can not serve as a direct source of reduced sulphur, since only bacteria, fungi and animals are able to synthesize cysteine from methionine via trans-sulphuration of cystathionine. Plants convert methionine to pyruvate, ammonia and methanethiol which is recycled by a S-alkyl exchange reaction with homocysteine to yield sulphide and recover methionine[18]. Sulphide can be fixed by OAS-TL and thus serve as reduced sulphur source. At higher concentrations of methionine, under our conditions at 3 mM and limiting sulphate but at 5 mM methionine during regular sulphate supply, the breakdown products apparently become toxic, indicated by release of volatile methanethiol[19], and plants die after longer exposure.

A supplement of 1.5 mM methionine led to an increase of cysteine and glutathione contents at both regular and minimal sulphate in the growth medium (figure 2). These rates, however, were lower than with cysteine feeding, and intracellular cysteine never exceeded glutathione concentrations. APS-K, SiR, and OAS-TL mRNAs were repressed by 30% to 50% in minimal sulphate medium but with a regular sulphate supply only SiR mRNA was similarly affected, while APS-K and OAS-TL remained essentially unchanged. The stronger repression in minimal compared to regular sulphate grown plants is probably due to the more effective breakdown of methionine in the sulphate starved plants which had to gain all required sulphur from this source.

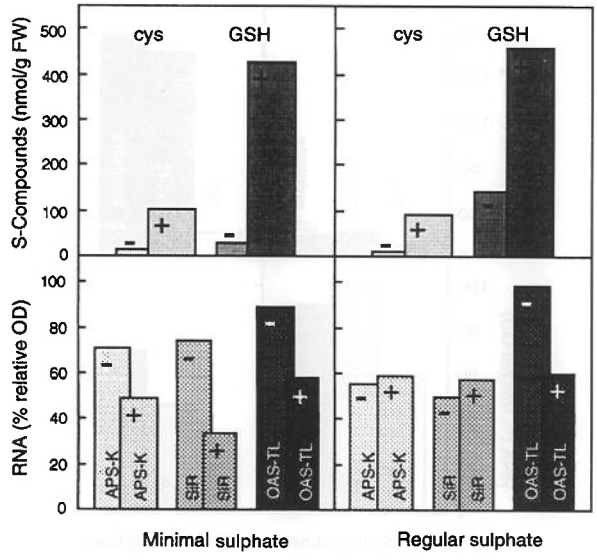


*Fig. 2.* Thiol and mRNA levels are affected by methionine application. Sterile *A. thaliana* plants grown on minimal (6 μM) and regular (2.5 mM) sulphate medium were supplemented with ("+") and without ("-") 1.5 mM methionine. Cysteine (cys), gluathione (GSH), and mRNAs of APS-K, SiR, and OAS-TL were determined as in table 1.

It is concluded that cysteine/methionine and sulphate supply have opposite effects on the expression of APS-K, SiR, and OAS-TL mRNA. Sulphate deficiency has a significant activating influence, whereas methionine and particularly cysteine repression seems to be of minor significance for the control of sulphate assimilation related gene expression. The genes analyzed exhibit a remarkable co-regulation in their mRNA contents that occurs on the basis of an apparently ubiquitous expression. It is proposed that the regulation of sulphate assimilation genes are triggered by a common signalling mechanism.

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# MOLECULAR EVIDENCE SUPPORTS AN APS-DEPENDENT PATHWAY OF REDUCTIVE SULPHATE ASSIMILATION IN HIGHER PLANTS

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## Abstract

Three different and novel *Arabidopsis thaliana* cDNAs, obtained by functional complementation of an *E. coli* cysH mutant defective in PAPS reductase activity to prototrophy, encode deduced proteins with significant homology to microbial PAPS reductases that also carry an N-terminal extension with the characteristics of a chloroplast transit peptide and a C-terminal thioredoxin-like domain. These PRH (**P**APS **R**eductase **H**omologue) proteins, representing new members of the thioredoxin superfamily, prefer APS over PAPS as substrate. We suggest that the thioredoxin domain is involved in catalytic function and that the PRH proteins may function as novel APS reductase enzymes. Our data support the idea that APS can be utilised directly, without activation to PAPS, as an intermediary substrate in reductive sulphate assimilation.

Sulphur in its reduced form plays an important role in plant metabolism, being involved in the biosynthesis of a wide range of primary and secondary S-containing metabolites. However the pathway of reductive assimilation of inorganic sulphate to sulphide in higher plants is the subject of some controversy[1,2]. After sulphate uptake, and its activation to 5' adenosine-phosphosulphate (APS) (5'-adenylylsulphate) by the enzyme ATP sulphurylase, it remains unclear whether APS is further metabolised by the enzyme APS sulphotransferase to "bound-sulphite" and then reduced to "bound-sulphide" by a thiosulphonate reductase or whether the PAPS-dependent "free-sulphite" pathway, found in most enterobacteria and certain fungi and yeast, operates. In this latter pathway APS is converted to 3'-phosphoadenosine-5'-phosphosulphate (PAPS) (3'-phosphoadenylyl-sulphate) by APS kinase, and PAPS is reduced to free sulphite by PAPS reductase. Sulphite is then reduced to sulphide by sulphite reductase. Demonstration of a thioredoxin-dependent PAPS reductase activity in spinach[3], as in *E. coli*, provides further support for the notion that the "free-intermediate" pathway operates not only in enterobacteria and yeasts but also in higher plants.

In order to examine this suggested role of PAPS reductase in higher plant reductive sulphate assimilation we attempted to clone *Arabidopsis thaliana* PAPS reductase cDNAs by functional complementation of the *E. coli cysH* mutant JM96, defective in PAPS reductase activity, to prototrophy with a cDNA library in the expression vector λYES[4]. The 57 complementing cDNA clones obtained after transfection of the λKC/JM96 lysogen with the λYES cDNA library could be sorted into three sequence-specific classes

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(characterised by Prh-19 (GenBank Accession Number: U53864), Prh-26 (GenBank Accession Number: U53865) and Prh-43 (GenBank Accession Number: U53866) (**P**APS **r**eductase **h**omologue)) by restriction digestion and partial sequencing[5].

The three cDNA sequences encode deduced proteins of $M_r$ 51,300, 50,500 and 50,453, respectively, that have 48-58% amino acid identity with fungal, yeast and bacterial PAPS reductases over the central region (residues 89-353 in the case of PRH-19) but also possess both an N-terminal and a C-terminal extension with respect to the microbial sequences. The central region of homology possesses four regions that are highly conserved between the PRH proteins and PAPS reductases including the conserved cysteine residue in the motif ECGLH (residues 347-351 of the PRH-19 sequence) and the conserved tyrosine residue in the motif GYxxG (residues 315-320 of the PRH-19 sequence) whose functional significance has been demonstrated by site-directed mutagenesis of the *E. coli* enzyme[6]. The N-terminal extension has the characteristics of a chloroplast transit peptide being rich in positively charged and hydroxylated amino acids[7]. The C-terminal extension has substantial homology with thioredoxin and suggests that the PRH proteins are new members of the thioredoxin superfamily. The homology includes the motif CxxC (at residues 385-388 of PRH-19) that carries the redox-active half-cysteine residues present at the active site of protein disulphide isomerase, thioredoxin and other related redox proteins[8].

Despite substantial homology between the PRH proteins and PAPS reductases the PRH proteins prefer APS over PAPS as substrate, and exhibit thioredoxin-independent activity, when tested in a conventional PAPS reductase assay (conversion of $^{35}$S-PAPS or $^{35}$S-APS to acid-volatile $^{35}$S-sulphite) in the presence of exogenous thioredoxin and with dithiothreitol as auxiliary reductant[9] (Table 1). The suggestion that the PRH proteins use APS as their *in vivo* substrate is supported by our observation that each of the Prh cDNAs are able to complement the *E. coli cysC* mutant JM81A, defective in APS kinase activity, to prototrophy. We note that there is no overall sequence similarity between APS kinase and the PRH proteins. To the extent of our present analysis the activity of the PRH proteins resembles that of APS sulphotransferase in that both enzymes prefer APS over PAPS and they are both thiol-dependent (data not shown) and chloroplast localised[1]. This suggests that at least part of the published data on APS sulphotransferase is a function of PRH proteins.

If this is the case then the presence of a thioredoxin domain in the PRH proteins, presumably catalytically functional but not yet tested, shows APS sulphotransferase in an entirely new light. Rather than acting as APS sulphotransferases the PRH proteins may act as APS reductases, perhaps in a manner analogous to that suggested for the *E. coli* thioredoxin-dependent PAPS reductase[6] but involving the internal thioredoxin domain rather than exogenous thioredoxin. The availability of affinity-purified recombinant PRH protein allows these suggestions to be tested. Our data provide further support for the suggestion that APS can be utilised directly, without activation to PAPS, as an intermediary substrate in reductive sulphate assimilation[1]. We note that none of the cDNAs isolated in the study reported here encode a "conventional" PAPS reductase.

Studies at the enzyme and/or transcript level show up-regulation of several steps of reductive sulphate assimilation, including APS sulphotransferase activity, on sulphate starvation. The steady-state transcript level of each of the Prh cDNAs described here also increases on sulphate starvation. This suggests to us that the PRH proteins described here are involved *in vivo* in sulphate assimilation and that they are part of the "stimulon" that is activated on sulphate starvation in an attempt to scavenge for available sulphur.

*Table 1.* PAPS reductase activity and APS reductase activity of the *cysH* mutant and the functionally-complemented and wild-type *Escherichia coli* strains. Cell-free extracts from the *cysH* mutant JM96, JM96 retransformed with either pYES, pPRH19, pPRH26 or pPRH43, and the wild-type strain TB1, were assayed for PAPS reductase activity[9] and APS reductase activity in the presence or absence of recombinant *E. coli* thioredoxin (trx) (4.5 μg)

| *E. coli* strain | PAPS reductase activity (pmol $min^{-1}$ mg $protein^{-1}$) | | APS reductase activity | |
|---|---|---|---|---|
| | + trx | - trx | + trx | - trx |
| TB1 (wildtype) | 482.7 | 26.8 | 0.9 | 1.0 |
| JM96 (*cysH* mutant) | 0.0 | 0.0 | 0.5 | 0.4 |
| JM96/pYES | 0.2 | 0.0 | 0.5 | 0.5 |
| JM96/pPRH19 | 1.3 | 1.9 | 601.7 | 588.0 |
| JM96/pPRH26 | 0.8 | 0.9 | 140.0 | 211.7 |
| JM96/pPRH43 | 1.1 | 1.4 | 732.5 | 766.5 |

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# ATP SULPHURYLASE OVER-EXPRESSION STUDIES IN TOBACCO PLANTS

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## Abstract

The complete cDNA sequence of an *Arabidopsis thaliana* ATP sulphurylase (ASA1) was inserted, in the sense orientation, under the control of the constitutive CaMV 35S promoter and before the NOS terminator in pBI 121. After transformation of tobacco plants with this construct, several plants which were identified as transgenic were self pollinated and characterised. ATP sulphurylase activities could be correlated with the ATP sulphurylase protein abundance, but not with the copy number of the transgene. The apparent molecular mass of the *Arabidopsis thaliana* ATP sulphurylase protein is lower than that expected when it is expressed in tobacco plants.

ATP sulphurylase is the first enzyme of the sulphur metabolism pathway. It catalyses the primary step of ATP-mediated activation of sulphate, generating adenosine 5'-phosphosulphate. We have cloned an *Arabidopsis thaliana* ATP sulphurylase cDNA (*ASA1*) by functional complementation of a yeast ATP sulphurylase mutant[1]. The complete coding region, as well as part of the 5' and 3' untranslated regions, of the ASA1 clone was inserted, in the sense orientation, between the cauliflower mosaic virus (CaMV) 35S promotor and the nopaline synthase (NOS) terminator of the plasmid pBI 121 (Clontech). A construct, using the complete pBI 121 plasmid containing the β-glucuronidase reporter gene, was used as a control (PBI 19 plant). Transgenic tobacco plants were produced with these constructs, using *Agrobacterium tumefaciens*-mediated leaf disc transformation. Eight tobacco plants which were kanamycin resistant were kept and analysed. The *ASA1* transformed plants did not present any obvious morphological modifications in the F1 generation, although chlorosis-like symptoms, suggesting sulphur deficiency, appeared on axillary leaves of some plants. Seven of the eight kanamycin resistant tobacco plants produced the expected 0.6 kb PCR product when their respective genomic DNAs were used to amplify a 0.6 kb *ASA1* fragment (Table 1-B). Among these plants, 4 showed a very significant increase in their *in vitro* ATP sulphurylase activities compared to the PBI 19 control plants (Table 1-A). There is no direct relationship between the ATP sulphurylase activity and the number of copies of the *ASA1* cDNA inserted in the tobacco genome (Table 1- A and C).

ATP sulphurylase activities were measured in all leaves of transformant ASA1-11 and found to be 3 to 5 fold higher than in control plant leaves, independant of their developmental stage (Figure 1). These high ATP sulphurylase activities correlated with an increased protein abundance in plants 11, 46, 47 and 93, as revealed by Western blot analysis using an *A. thaliana* anti-ATP sulphurylase (APS3)[2] serum (Table 1-D). In these 4 transformed plants, as well as in a control tobacco (*Nicotiana plumbaginifolia)* plant, we found that the apparent molecular weight of the protein recognized by the

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APS3 ATP sulphurylase antibody is 4 to 7 kDa lower than the predicted 48 kDa for *A. thaliana* mature protein[1,3]. These results suggest that when the *ASA1 A. thaliana* protein is expressed in tobacco, the protein is processed, but the truncated protein is still active in *in vitro* assays.

*Table 1.* Characterisation of *ASA1* transformed tobacco plants
**A**: ATP sulphurylase activities were measured by molybdolysis[4]. The results, which are the means of 3 to 5 replicates, are expressed in nmol Pi $min^{-1}$ $mg^{-1}$ protein.
**B**: Purified genomic DNA from each tobacco plant was used as PCR template with the appropriate synthetic oligonucleotides: + indicates the amplification of the expected 0.6kb *ASA1* product.
**C**: The *ASA1* copy number in each transgenic plant was determined after restriction digestion of the genomic DNA, and Southern blot analysis.
**D**: ATP sulphurylase protein abundance was determined by Western blot analysis using an *A. thaliana* ATP sulphurylase (APS3) antibody kindly provided by Dr Thomas Leustek (Rutgers University, USA). The proteins were detected by luminescence using the ECL Western blotting system (Amersham)
nd: not determined, - not detected, and + present, N t Not transformed tobacco plant

| | ASA1 Transformed plants | | | | | | | | Control plants | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 11 | 34 | 44 | 46 | 47 | 70 | 92 | 93 | PBI 19 | N t |
| **A**- Activity | 1770 | 17 | 23 | 1962 | 195 | 63 | 13 | 188 | 70 | / |
| **B**- PCR product | + | + | + | + | + | - | + | + | - | - |
| **C**- Copy number | 1 | 1 | 2 | nd | 2 | 0 | 3 | nd | nd | 0 |
| **D**- Abundance | +++ | ++ | ++ | +++ | +++ | + | ++ | ++ | ++ | + |

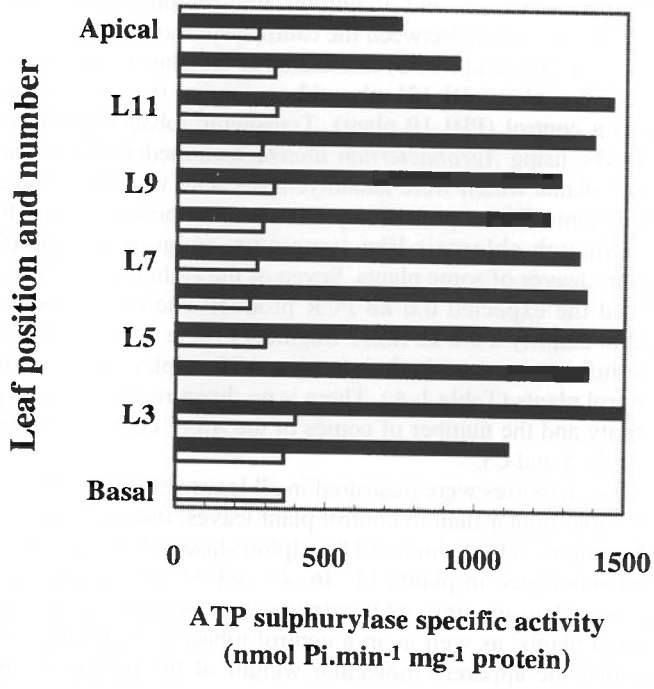


Fig. 1. ATP sulphurylase activities in six week old tobacco, control PBI 19 (□) or ASA1-11 (■) transgenic plants. ATP sulphurylase activities were measured in leaves of different ages harvested at different positions. The oldest, basal, leaf is numbered (L1) and youngest, apical, leaf is referred to as L13. ATP sulphurylase activities were measured in duplicate, on the same sulphate-free dialysed fraction, by molybdolysis[4] at 37°C.

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# GENETIC CONTROL OF SULPHITE PRODUCTION IN BREWER'S YEAST

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## Abstract

Overproduction of sulphite from *S. carlsbergensis*, brewer's yeast, can be genetically controlled by disrupting either the *MET2* or the *MET10* genes in this yeast. Overexpression of the *MET3, MET14* and *MET16* genes only had a minor effect on sulphite production.

In yeast sulphur assimilation, sulphate is reduced to sulphite, an intermediate in the formation of methionine and cysteine. Sulphite is a key compound for flavour stability of beer[2,5,6]. For the purpose of genetic control of sulphite production by brewer's yeast, the effects of decreasing or increasing gene activities in the sulphur assimilation pathway have been studied.

In *Saccharomyces* yeasts, the gene products of *MET3*, *MET14* and *MET16*, ATP sulphurylase, APS kinase and PAPS reductase, respectively, are responsible for the reduction of sulphate to sulphite (fig. 1). Sulphite is further reduced to hydrogen sulphide via the action of sulphite reductase, for the formation of which the genes *MET1, MET5, MET8, MET10* and *MET20* are responsible. Homoserine acetyltransferase, encoded by *MET2*, condenses *O*-acetyl homoserine and hydrogen sulphide to form homocysteine, which is precursor of cysteine, methionine and *S*-adenosylmethionine. Most genes in the pathway are transcriptionally repressed when *S*-adenosylmethionine is added[3,7]. The flux through the pathway is consequently also controlled by the presence of methionine in the growth medium.

Three different strategies were applied in order to control the sulphite production. The genes *MET2* (fig. 2) and *MET10* (fig. 3) were partially or completely inactivated in *Saccharomyces carlsbergensis* brewer's yeast, while *MET3, MET14* and *MET16* were overexpressed from chimeric gene constructs integrated chromosomally in *Saccharomyces cerevisiae* (fig. 4).

Disruption of *MET2* leading to a block in homoserine acetyltransferase, was predicted to eliminate $H_2S$ usage and formation of *S*-adenosylmethionine (SAM) from sulphate, resulting in diminished transcriptional repression of sulphur assimilation genes normally seen when high levels of *S*-adenosylmethionine are present. Strains with reduced *MET2* activity, showed a significant increase in hydrogen sulphide production (data not shown). We think product inhibition of the sulphite reductase by hydrogen sulphide is the reason why sulphite also accumulates within these strains (fig. 2). Sulphite production increases when three *MET2* genes are inactivated (fig. 2), and inactivation of the fourth copy results in further increase.

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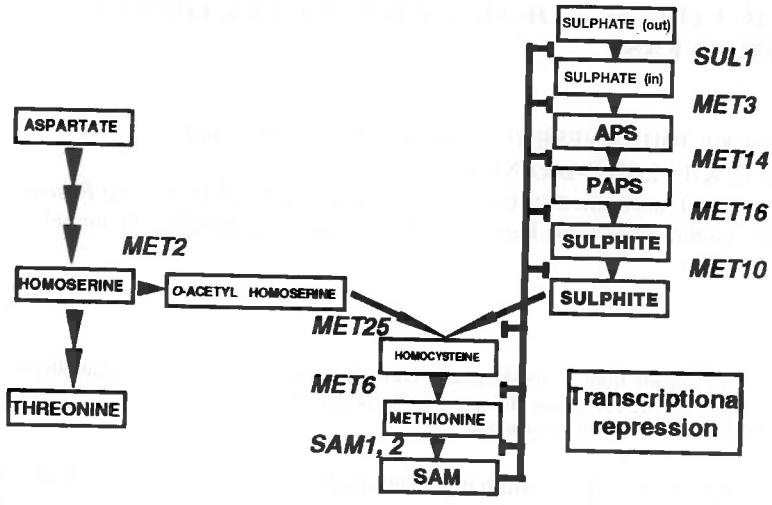


*Fig. 1.* Sulphur assimilation in yeast. APS: Adenylylsulphate; PAPS: Phosphoadenylylsulphate; SAM: *S*-adenosylmethionine.

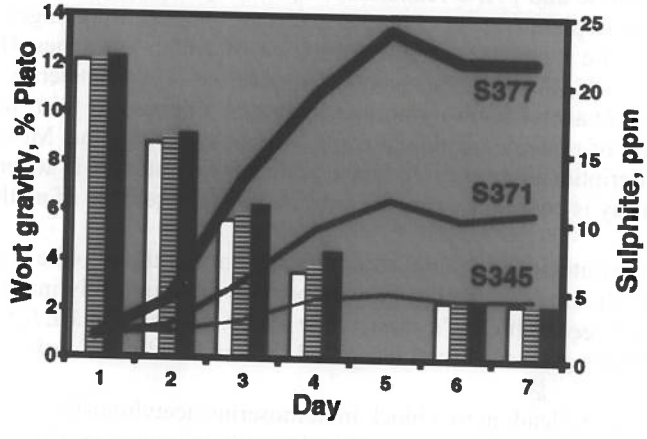


*Fig. 2.* Sulphite production (lines) and fermentation profile (bars) from 2 l brewer's wort fermented with the various strains for 7 days at 13°C. Control strain S345 (thin line/white bars), strain S371 (medium line/striped bars, contains one active *MET2* gene, three copies have been disrupted), strain S377 (thick line/black bars, all four copies of *MET2* have been disrupted). Sulphite was measured by the pararosanilin-method[4].

Disruption of *MET10* supposedly will result in a nonfunctional sulphite reductase. This was believed to cause derepression of the sulphur assimilation genes by the mechanism discussed for disruption of *MET2*. Sulphite should accumulate immediately as it cannot be reduced. Disruption of all four *MET10* copies results in a strong increase in sulphite production (fig. 3.), whereas hydrogen sulphide production is abolished in strain SB130 (data not shown).

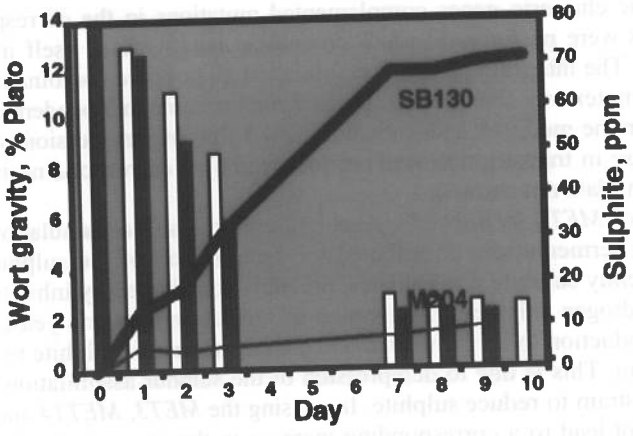


*Fig. 3.* Sulphite production (lines) and fermentation profile (bars) of strain M204 (thin line/black bars, parental strain with four wild type copies of *MET10*) and SB130 (thick line/white bars, disrupted in all four copies of *MET10*) during an 11 day fermentation of brewer's wort in 50 l fermentation vessels at 13°C.

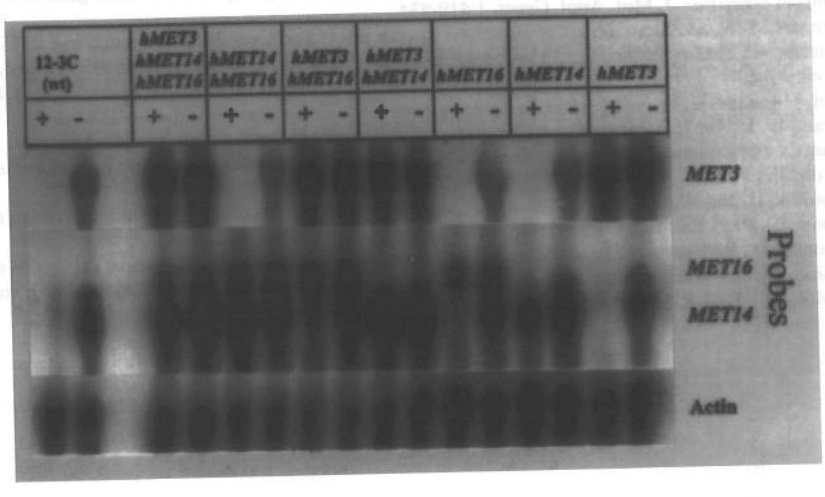


*Fig. 4.* Northern blot of total RNA of transformants obtained by integration grown in minimal medium with 0.2 mM homocysteine as sulphur source with (+) or without (-) 2 mM L-methionine (repressing amount). 10 µg of total RNA was electrophoresed in each lane in a 1% agarose gel. The RNA was transferred onto a nylon membrane. The RNA's were probed with radioactively labelled fragments of the coding region of *MET3, MET14* and *MET16*. The actin probe was used as a loading control. The signals were detected using a Phosphor Imager (Molecular Dynamics).

The last strategy involves overexpression of *MET3, MET14* and *MET16,* and removal of their wild-type regulation. This could lead to further sulphite accumulation. The *TPI* (Triose Phosphate Isomerase) promoter[1] was fused to the coding region of *MET3, MET14* and *MET16*. Consequently, the three chimeric genes were expected to be independent of regulation by the presence of *S*-adenosylmethionine, making overexpression

possible. All the chimeric genes complemented mutations in the corresponding genes. All integrations were performed in a *S. cerevisiae met10* strain, itself impaired in sulphite reduction. The integrations were performed in all possible combinations. Whenever a wild type promoter was changed, the transcription became independent of the presence of methionine in the medium, and, moreover, a 4-5 fold overexpression was seen. However, the increase in transcription was not followed by a corresponding increase in sulphite production (data not shown).

Inactivation of *MET2* in brewer's yeast causes increased accumulation of hydrogen sulphide during fermentation, contributed by derepression of the sulphur assimilation genes. Subsequently sulphite accumulates, probably contributed by inhibition of sulphite reductase by hydrogen sulphide. Inactivation of *MET10* in brewer's yeast causes hydrogen sulphide production to be eliminated and a large amount of sulphite to be accumulated in the medium. This is due to derepression of the sulphur assimilation genes and the inability of this strain to reduce sulphite. Increasing the *MET3, MET14* and *MET16* transcription does not lead to a corresponding increase in the amount of sulphite produced. Thus, none of these enzymatic steps seems to form a bottleneck in the pathway.

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# REGULATION OF ATP SULPHURYLASE ENZYME ACTIVITY

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## Abstract

Three plant cDNAs were isolated by functional complementation of ATP sulphurylase deficient yeast mutants. In protein extracts of yeast cells expressing the plant cDNAs ATP sulphurylase activity was detected. The enzyme activity of the plastidic ATP sulphurylase AtMET3-1 is inhibited by glutathione whereas the activity of the cytosolic enzyme StMET3-1 is not affected.

Plants are able to meet their total sulphur requirement by reducing inorganic sulphate. After uptake from the soil sulphate is activated by the enzyme ATP sulphurylase, which leads to the formation of 5'-adenosine-phosphosulphate (APS). We identified two cDNAs encoding ATP sulphurylases from potato via functional complementation of a yeast mutant. The deduced amino acid sequences of the potato ATP sulphurylases indicate that one enzyme is localized in the plastids (StMET3-2) whereas the other isoform is cytosolic (StMET3-1)[1]. In addition, a putative plastidic ATP sulphurylase (AtMET3-1) from *Arabidopsis thaliana* was isolated by functional yeast complementation[2].

Protein extracts of yeast cells expressing the plant ATP sulphurylases were biochemically analyzed. ATP sulphurylase activity was measured in a coupled assay as described by Burnell[3]. In yeast strains expressing the plant cDNAs ATP sulphurylase activity was clearly demonstrated (Table 1). It is possible that the observed variation in activity level was caused by problems with the folding and processing of individular plant proteins in the yeast cells. To analyze the regulation of plant ATP sulphurylases the cytosolic ATP sulphurylase StMET3-1 and the plastidic enzyme AtMET3-1 were used for *in vitro* inhibitor studies. ATP sulphurylase activity was not influenced by $SO_3^{2-}$, $S_2O_3^{2-}$, $ClO_4^-$, $NO_3^-$, sodium-selenate, potassium-chromate, 3'-phospho-5'-adenosine-phosphosulphate (PAPS), cysteine, methionine, serine, homoserine and cystathionine at a concentration of 0.5mM. However, the plastidic enzyme AtMET3-1 was inhibited by *S*-adenosylmethionine (SAM), *O*-acetylserine (OAS) and glutathione (GSH), whereas the cytosolic ATP sulphurylase StMET3-1 was unaffected. The effect of different concentrations of SAM, OAS and GSH on the ATP sulphurylase activity of AtMET3-1 and StMET3-1 is shown in Fig. 1. The inhibitory effect of *S*-adenoyslmethionine and *O*-acetylserine was only detected at concentrations (0.5-2.5mM) which are presumably not present in plant tissues. The inhibition of the plastidic ATP sulphurylase AtMET3-1 by glutathione could have physiological relevance. Glutathione at concentrations of 0.5mM and 2.5mM caused inhibition of AtMET3-1 of 63% and 86% respectively. In chloroplasts of spinach a glutathione concentration of 2.33 mM was measured[4], suggesting the possibility that the plastidic ATP sulphurylase is subjected to significant inhibition *in vivo*.

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*Table 1.* ATP sulphurylase activities in protein extracts from yeast wild type (X2180), ATP sulphurylase mutants (W303met3-1B[1], W303met3-7D[2]) and the mutant strains expressing a plant ATP sulphurylase. The average value measured in three different cultures is shown

| strain | activity (nmol $min^{-1}$ mg $protein^{-1}$) | activity (%) |
|---|---|---|
| X2180 | 289 ± 89 | 100 |
| W303met3-1B | 32 ± 15 | 11 |
| W303met3-7D | 29 ± 16 | 10 |
| W303met3-1B + pStMET3-1 | 981 ± 322 | 339 |
| W303met3-1B + pStMET3-2 | 91 ± 44 | 31 |
| W303met3-7D + pAtMET3-1 | 1167 ± 103 | 404 |

Using a cytosolic ATP sulphurylase (StMET3-1) and a plastidic ATP sulphurylase (AtMET3-1) it was shown that the enzymes from the different compartments are differently regulated. This result supports the hypothesis that sulphate activation in the cytosol and in the plastid has different physiological functions. The plastidic ATP sulphurylase is presumably involved in assimilation of sulphate into amino acids and other thiols. It was shown that glutathione, the most abundant soluble thiol of plants, inhibits sulphate uptake[5, 6, 7], most likely at the level of gene expression. Here we have demonstrated for the first time that glutathione act as a direct inhibitor of plastidic ATP sulphurylase.

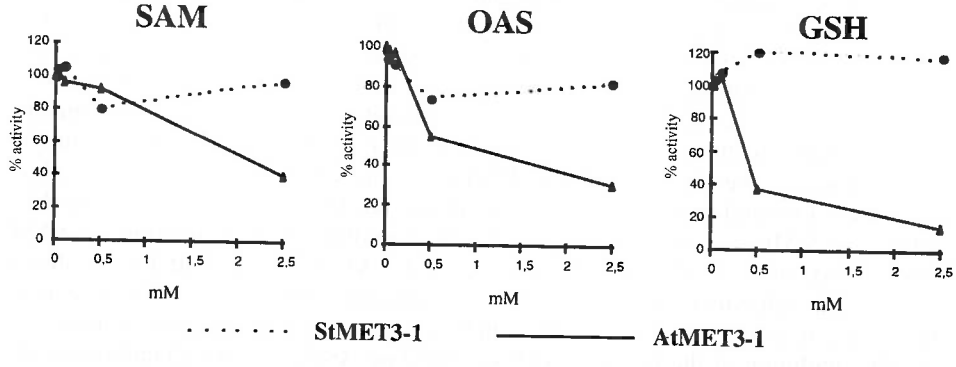


*Fig. 1.* ATP sulphurylase activities determined in yeast protein extracts following preincubation with different concentrations of S-adenosylmethionine (SAM), *O*-acetylserine (OAS) and glutathione (GSH). The results are expressed as a % of the control value without preincubation; the mean of two measurements for each point is shown.

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# COMPARISON BETWEEN DEMAND-DRIVEN REGULATION OF ATP SULPHURYLASE ACTIVITY AND RESPONSES TO OXIDATIVE STRESS

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## Abstract

Nutritional stress caused by S starvation results in a decrease in internal glutathione, and an increase in ATP sulphurylase activity and $SO_4^{2-}$ uptake. Considering that glutathione pools are known to vary in response to oxidative stress, we question here whether this regulation of sulphur metabolism in roots was triggered off by an oxidative stress. The effects of oxidative stress (10 mM $H_2O_2$ added in culture solution) on GSH, GSSG, ATP sulphurylase activity, and $SO_4^{2-}$ uptake in canola are compared to those of nutritional stress (sulphate withdrawn from culture solution)[2]. The activity of ascorbate peroxidase, an enzyme involved in the $H_2O_2$ scavenging pathway, has also been measured in $H_2O_2$-treated and sulphate-starved plants. The results are discussed in relation to the role of GSH as regulatory signal for demand-driven control of the root ATP sulphurylase activity.

The activity of ATP sulphurylase extracted from roots of intact canola (*Brassica napus* L., cv Drakkar) is enhanced by withholding the S source ($SO_4^{2-}$) from nutrient solution, and declines when S-starved plants are replaced in $SO_4^{2-}$-containing solution[1]. In these plants, $SO_4^{2-}$ uptake was similarly influenced by nutritional stress, as observed in other species[2,3,4]. These demand-driven processes involve a remote control mediated by phloem-translocated glutathione that provides information concerning the nutritional status of the leaves to the roots[1]. The observed variations of the glutathione pools in root tissues and in sieve sap of S-starved plants, and the effects of GSH treatments on ATP sulphurylase activity and $SO_4^{2-}$ uptake, support this hypothesis. Internal glutathione is known to be related to oxidative stress, and oxidative stress responses[5]. This study aimed at determining whether the identified responses of ATP sulphurylase activity and $SO_4^{2-}$ uptake to nutritional stress (S starvation) were due to specific demand-driven regulation or to the effects of an oxidative stress triggered off by S starvation.

Oxidative stress was experimentally induced by feeding plants with 10 mM $H_2O_2$ for 4 to 8 h. Though internal $H_2O_2$ was not measured in $H_2O_2$-treated plants, it has been established that oxidative stress-like responses[5,6] occurred. This treatment led to a rapid decline in root [GSH], down to an undetectable level within 4 h, and a slight increase in [GSSG], which did not compensate for [GSH] (Table 1). The *in vitro* activity of ATP sulphurylase extracted from roots was stimulated by oxidative stress (Fig. 1), as it was by nutritional stress[1]. Since phloem-translocated glutathione is supposed to be responsible for regulation of ATP sulphurylase activity, we measured [GSH] and [GSSG] in phloem exudates from plants treated with $H_2O_2$ for 8 h. Both concentrations decreased, but significant concentrations of GSH were still found (ca. 15% compared to control plants).

S deprivation for 1 to 3 d led to decline in [GSH] and increase in [GSSG] (Table 1), resulting in decreased total glutathione concentration (30% after 3 d of S-starvation) and

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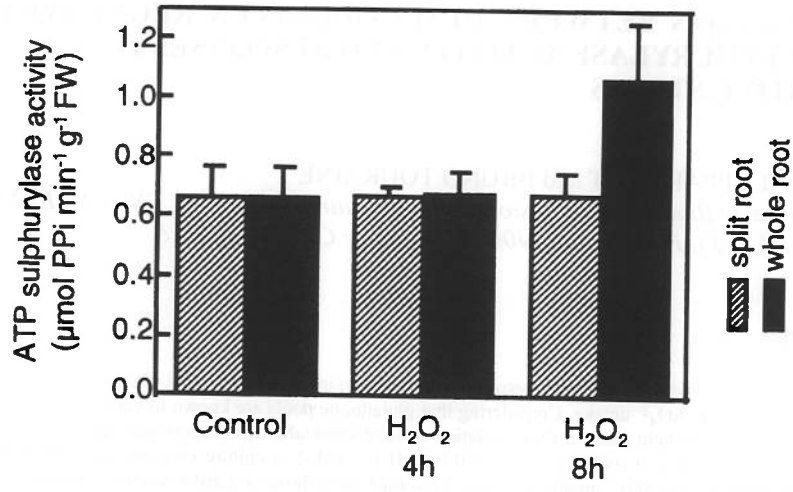


*Fig. 1.* Effect of 10 mM $H_2O_2$ on ATP sulphurylase activity in canola roots. Extracts for enzymatic assay were obtained by tissue homogenization in 100 mM Tris-HCl pH 8, 10 mM sodium-EDTA, 2 mM DTT and centrifugation for 15 min at 16,000 g. Enzyme activity was determined according to molybdolysis assay[12]. $H_2O_2$ was given either to all the roots ("Whole root"), or to part of the roots ("Split root"), measurements then being made in the other roots. Mean (5 replicates) ± 95% confidence limits.

8-fold decreased GSH/GSSG. We[1] have previously shown that feeding plants with 1 mM GSH and S starvation had the opposite effect on ATP sulphurylase activity, as expected from the respective changes in internal [GSH]. This GSH treatment resulted in similar increases in [GSSG] and [GSH] (Table 1).

The known relation between ascorbate peroxidase activity and oxidative stress response[9,10,11], was used as an oxidative stress marker. The ascorbate peroxidase activity is similarly enhanced by S starvation and $H_2O_2$ treatment (Fig. 2). The response of this activity to $H_2O_2$ was quasi-immediate. By contrast, the effect of S starvation was not observed within the first day, whereas ATP sulphurylase activity significantly increased within this period[1]. These differences in the response patterns of activities of ATP sulphurylase and ascorbate peroxidase to nutritional and oxidative stresses suggest that the effects of S starvation are not mediated by an oxidative stress. Furthermore, the GSH treatment strongly stimulated ascorbate peroxidase activity (15-fold increase within 24 h, Fig. 2), while ATP sulphurylase activity was inhibited[1].

*Table 1.* Effects of withholding sulphate from the nutrient solution for 3 d, or adding 1 mM GSH for 1 d or 10 mM $H_2O_2$ for 8 h on glutathione pools in root tissues from intact canola plants. Glutathione was extracted with 0.1 N HCl, 1mM sodium-EDTA and quantified by HPLC-analysis after reduction with DTT and derivatization with monobromobimane[1]. GSSG was measured after addition of N-ethylmaleimide to the extracts in order to prevent GSH derivatization due to binding to thiol group[8]

| Treatment | total Glutathione | GSH | GSSG | GSH/GSSG |
|---|---|---|---|---|
| | | *nmol/g FW* | | *mol/mol* |
| control | 1269 ± 190 | 1124 ± 175 | 152 ± 29 | 7.9 ± 1.4 |
| -S 72 h | 787 ± 192 | 287 ± 128 | 468 ± 142 | 0.8 ± 0.5 |
| + GSH 24 h | 4477 ± 873 | 3630 ± 885 | 506 ± 84 | 6.0 ± 2.0 |
| + $H_2O_2$ 8 h | 754 ± 158 | not detected | 702 ± 128 | 0 |

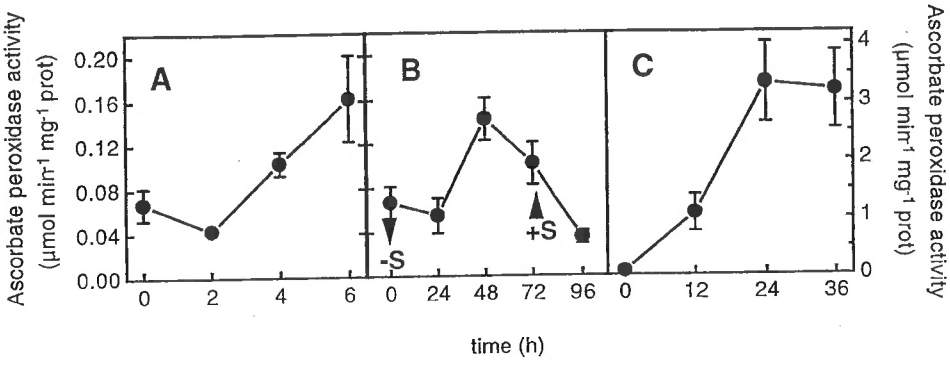


*Fig. 2.* Effects of oxidative stress (A), $SO_4^{2-}$ availability (B), and GSH (C) on ascorbate peroxidase activity extracted from roots of 21-d-old *Brassica napus*. A, 10 mM $H_2O_2$ added at time 0; B, S withheld (-S) for various periods as indicated, or withheld for 72 h and resupplied (+S) for 24 h, prior to root harvest; C, 1 mM GSH added at time 0. Extracts prepared as in Fig. 1. Enzyme activity determined photometrically at 285 nm[13]. Mean (5 replicates) ± 95% confidence limits.

Overall, nutritional and oxidative stresses alter the pools of GSH and GSSG, and ATP sulphurylase activity, in the same ways. These changes may thus be responsible for the observed response of ATP sulphurylase activity in roots from S starved plants. Considering the effects of both stresses on ascorbate peroxidase, it is unlikely, however, that the response of ATP sulphurylase to S starvation is mediated by an oxidative stress. In addition to its known involvement in oxidative stress response, the role of glutathione, probably as GSH, in the shoot-root signaling of nutritional status is reinforced by this study, where in every treatment applied – S starvation, GSH, $H_2O_2$ – a negative correlation between GSH and ATP sulphurylase activity was observed.

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# ARE ATP SULPHURYLASE mRNA AND PROTEIN ACCUMULATED IN ROOTS OF *ARABIDOPSIS* FOLLOWING S STRESS?

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## Abstract

In *Brassica napus*, the activity of ATP sulphurylase extracted from roots depends upon the nutritional status of the whole plant. Phloem-translocated GSH is likely to be the shoot-to-root message involved in this demand-driven regulation[1]. In order to identify the underlying mechanism operating in roots, time-course responses of mRNA and protein levels of ATP sulphurylase to modifications of S nutritional status were analyzed. This study was performed in *Arabidopsis thaliana*, where specific cDNAs are available[2,3]. Overall, the data obtained show that both the mRNA and protein levels are affected by nutritonal status in the same way as the enzyme activity.

The activity of ATP sulphurylase extracted from roots of intact *Brassica napus* was enhanced by withholding the S source ($SO_4^{2-}$) from nutrient solution, and declined when S-starved plants were replaced in $SO_4^{2-}$-containing solution[1]. Variations of internal GSH correlated with the ATP sulphurylase responses to S availability. Moreover, increasing internal GSH by feeding plants with this compound inhibited ATP sulphurylase activity. Based on these observations and other evidence, we hypothesized that GSH is responsible for mediating the responses to S availability. The underlying mechanisms are not known. Especially, the question arises whether this regulation corresponds to a variation in ATP sulphurylase protein levels. Then, if the protein level appeared to respond to nutritional stress, the question of the level at which this control is exerted (transcriptional or translational) is raised. These two questions were addressed using cDNA and antibodies obtained with *Arabidopsis thaliana*. As a first step, we checked that a demand-driven regulation, similar to that described for *Brassica napus*, is operating in this species.

Plants of *Arabidopsis thaliana*, ecotype Columbia, were grown hydroponically in sterile conditions, on a culture solution containing all required mineral nutrients and 10 g $l^{-1}$ sucrose at pH 5.7. The activity of ATP sulphurylase, extracted from roots of 21-d-old plants, significantly increased upon feeding with a $SO_4^{2-}$-free, otherwise identical, solution for 1 to 3 d prior to harvest, and decreased upon transfer of S-starved plants to S-replete solution (Fig 1A). 1 mM GSH inhibited ATP sulphurylase activity by 40% within 12 h (Fig 1B). Overall, these response patterns are identical to those observed in *Brassica napus*[1], though the quantitative impact of S starvation was lower, perhaps because of the presence of sucrose in the culture media.

One of the ATP sulphurylase cDNA cloned in *Arabidopsis*, *APS3*, has been used to purify a recombinant enzyme[3]. Antibodies raised against APS3 have been obtained at Rutgers University and were used in Montpellier to immunoblot proteins from root extracts. A single band was visualized at approximately 50 kDa. The relative protein level increased within 24 h S-starvation (Fig 2). Conversely, it sharply declined when plants previously

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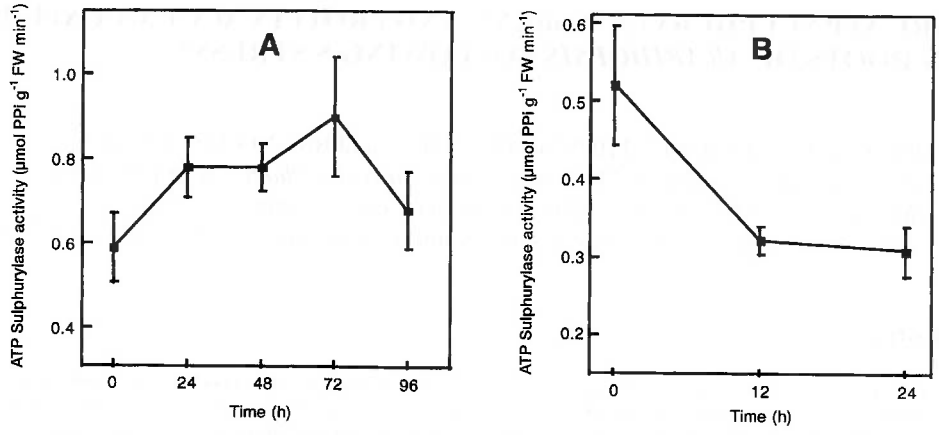


*Fig. 1.* Effects of S starvation and refeeding (A), and GSH treatment (B) on the activity of ATP sulphurylase extracted from roots of *Arabidopsis*. Plants were grown on a complete nutrient medium, including 1.5 mM $SO_4^{2-}$, for up to 21 d (date of harvest). A, $SO_4^{2-}$ was withdrawn from the culture solution 0 to 3 d before harvest (0 to 72 h on X axis), or withdrawn for 3 d and then resupplied for 1 d (96 h on X axis); B, 1 mM GSH was added into culture solution at time 0. Extracts for enzymatic assay were obtained by tissue homogenization in 100 mM Tris-HCl pH 8, 10 mM sodium-EDTA, 2 mM DTT and centrifugation for 15 min at 16,000 g. ATP sulphurylase activity was determined as the molybdate-dependent formation of pyrophosphate[4]. Bars represent 95% confidence limits (5 replicates).

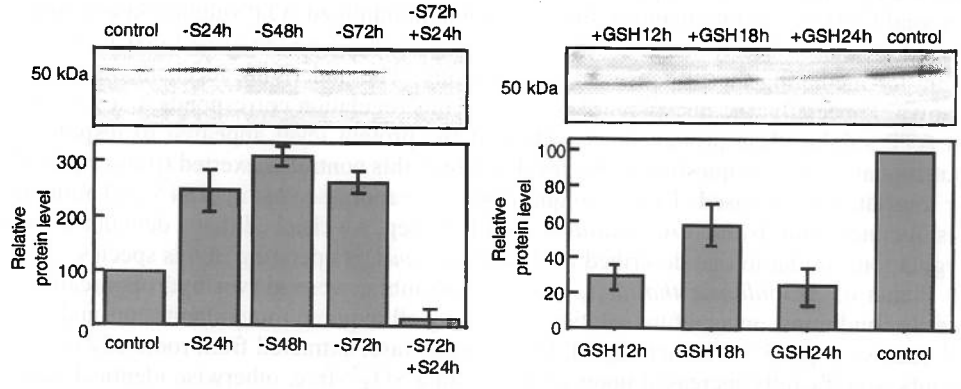


*Fig. 2.* Effects of sulphate availability (left), and GSH treatment (right) on relative abundance of ATP sulphurylase protein in crude extracts from roots of *Arabidopsis*. -S, withdrawal of sulphate from the culture solution; +S, resupply with 1.5 mM sulphate; +GSH, addition of 1 mM GSH in culture solution. The roots were collected at day 21, following periods of treatments as indicated, and proteins were extracted as in figure 1. Upper panels: western blots from gels loaded with 5 µg total proteins per lane. The polypeptides were separated on 10% SDS-PAGE and transferred on nitrocellulose membrane. ATP sulphurylase was detected with serum raised in rabbit against the APS3 polypeptide[3], and visualized by chemiluminescence (Amersham). The blots presented are representative of three individual experiments. Bottom panels: relative level of the APS3 immuno-detected protein derived from blots scanning (bars, sd).

starved of S for 3 d were re-supplied with $SO_4^{2-}$ for 24 h. Changes in relative protein levels are thus consistent with response of enzyme activity to S availability. Adding 1 mM GSH to the culture solution resulted in a reduction of the ATP sulphurylase protein level (Fig 2). Furthermore, this treatment also led to a decrease in the accumulation of mRNA hybridizing with *APS1* cDNA (Fig 3).

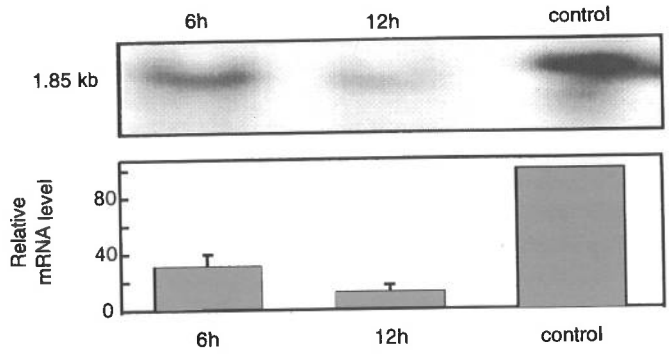


*Fig. 3.* Effect of 1mM GSH (time indicated on figure) on ATP sulphurylase mRNA level in roots of *Arabidopsis*. Culture as in figure 1. Roots were collected at day 21, following periods of treatment as indicated. Total RNA were isolated, from which 20 µg were separated on 0.66 M formaldehyde gel, transferred to Hybond N membrane (Amersham), and probed with $^{32}$P-labelled *APS1* cDNA. Upper panel: blot representative of three individual experiments. Bottom panel: relative level of the *APS1* probed mRNA derived from blots scanning (bars, sd).

This study reveals that a transcriptional regulation of ATP sulphurylase is operating in roots of *A. thaliana* in response to changes in internal GSH levels. The changes in mRNA accumulation can seemingly account for the observed variations in protein levels. One cannot dismiss, however, the possibility that other controls may be exerted on the activity of ATP sulphurylase. The compatibility between observed responses to S starvation and GSH treatment is consistent with the GSH-dependent regulation being responsible for the demand-driven control of ATP sulphurylase activity. The transduction pathway between GSH and the transcript levels, which remains to be elucidated, may involve other compounds, possibly a GSH metabolite. Nevertheless, the identification of this compound as the phloem-translocated molecule responsible for the responses of ATP sulphurylase activity and $SO_4^{2-}$ uptake in root[1] ascribes to GSH the role of the inter-organ signal involved in the genes expression regulation.

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# MOLECULAR GENETICS OF SULPHONUCLEOTIDE REDUCTION IN HIGHER PLANTS

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## Abstract

To identify the components involved in sulphate reduction in higher plants a functional complementation method was used to clone three cDNA's from *Arabidopsis thaliana* that are able to transform a *cysH*, 3'-phosphoadenosine-5'-phosphosulphate (PAPS) reductase mutant strain of *Escherichia coli* to cysteine prototrophy. Sequence analysis revealed that the clones may encode plastid-localized, bifunctional enzymes consisting of a PAPS reductase domain and a thioredoxin-like domain. These clones were found to also be able to complement a cysC, APS kinase mutant of *E. coli*. The results suggest that the enzyme encoded by the APR clones may use APS as a substrate.

Plants, like other sulphate assimilating organisms, reduce inorganic sulphate for cysteine biosynthesis. There has been some controversy, however, about the mechanism they use. Figure 1 shows the proposed pathway in plants compared with that in enteric bacteria. Plants and bacteria activate sulphate by forming 5'-adenylylphosphosulphate (APS). After this common step plants are thought to transfer sulphate from APS to a thiol compound, possibly glutathione (GSH), to form a thiosulphonate[1]. APS sulphotransferase, the enzyme catalyzing this reaction can also use a variety of reduced thiol compounds. The thiosulphate formed is converted to sulphide by a reductase and then sulphide is incorporated into cysteine. The evidence for APS sulphotransferase comes primarily from metabolic studies with cell-free extracts.

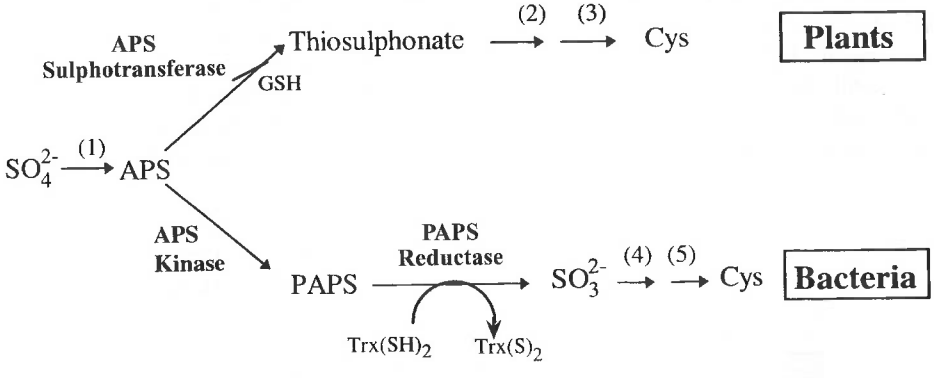


*Fig. 1.* Sulphate Reduction in Plants and Bacteria. The enzymes that pertain directly to this paper are indicated in bold type. GSH refers to reduced glutathione, $Trx(SH)_2$ refers to reduced thioredoxin and $Trx(S)_2$ to oxidised thioredoxin. Other reactions include, (1) ATP sulphurylase, (2) thiosulphonate reductase, (3) cysteine synthetase, (4) sulphite reductase-cysteine synthetase.

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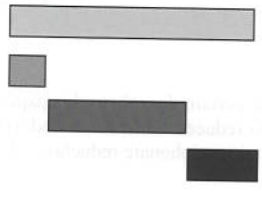
In enteric bacteria, the sulphate reduction pathway was defined by analysis of Cys auxotrophic mutants[2]. In these organisms, APS is phosphorylated to PAPS by APS kinase. Sulphite is then formed through the action of PAPS reductase, a thioredoxin-dependent enzyme. Finally, sulphite is reduced to sulphide and is then incorporated into cysteine.

Since its discovery, the existance of APS sulphotransferase has been controversial, yet this hypothesis for the pathway of sulphate reduction in higher plants has been widely cited in plant biochemistry text books. The physiological characteristics of this enzyme point toward a direct role in sulphate reduction[3]. Alternatively, a bacterial-type pathway is evidenced by the existance in plants of APS kinase and sulphite reductase. More recently it was reported that plant APS kinase can catalyze an APS sulphotransferase-like reaction[4]. Thus, there are significant, unanswered questions remaining about the sulphate reduction pathway in higher plants[5].

Part of the difficulty in studying sulphate reduction through biochemical means is that the pathway intermediates are highly reactive and subject to side reactions. Moreover, the enzymes are unstable and difficult to purify. Recent successes in cloning of genes for sulphate assimilation enzymes from higher plants by functional complementation suggested a method for identifying the genes for sulphate reduction enzymes. This method was used to identify cDNAs from the higher plant *A. thaliana* that can substitute for the PAPS reductase of *E. coli*. Approximately 1 million independent transformants were screened for the ability to complement a *cysH* mutant strain of *E. coli* (JM96), to cysteine prototrophy. Twenty four independent clones were isolated comprising 3 classes of cDNA, named *APR1*, *2* and *3* (Arabidopsis PAPS Reductase). The coding sequences of the *APR* clones all share the characteristics illustrated for *APR1* in figure 2.

All the APR clones encode polypeptides with amino terminal transit peptides for localization to plastids. The central region of each is homologous with *E. coli* PAPS reductase, and the carboxyl-terminal regions are homologous to disulphide exchange enzymes like thioredoxin and protein disulphide isomerase. The homology with PAPS reductase and thioredoxin is particularly striking in two highly conserved regions that have been shown to be important for activity of these enzymes[6,7] (fig. 3). For example, the Tyr and Cys residues that were shown to be important in *E. coli* PAPS reductase[6] are conserved in yeast PAPS reductase and *APR1*. The active site of thioredoxin is well conserved, particulalry the Trp and vicinal Cys residues. In addition, *APR1* contains a helix-breaking Pro between the Cys residues, a feature of thioredoxin active sites.

In total, these results suggest that *A. thaliana* contains an enzyme that is similar to PAPS reductase of microorganisms. The presence of a thioredoxin-like domain might be the result of evolutionary convergence of the two components found in microorganisms



| | Amino Acids | Percent Homology |
|---|---|---|
| *APR1* | 455 | - |
| transit peptide | 75 | - |
| PAPS Reductase | 243 | 48% |
| Thioredoxin | 127 | 53% |

*Fig. 2.* Domains of *APR1*. The domains are shown relative to the full length translation product. To the right are shown the size of each domain in amino acids, and the homology of each domain with *E. coli* PAPS reductase and mouse protein disulphide isomerase. The GenBank accession number for *APR1* is U43412.

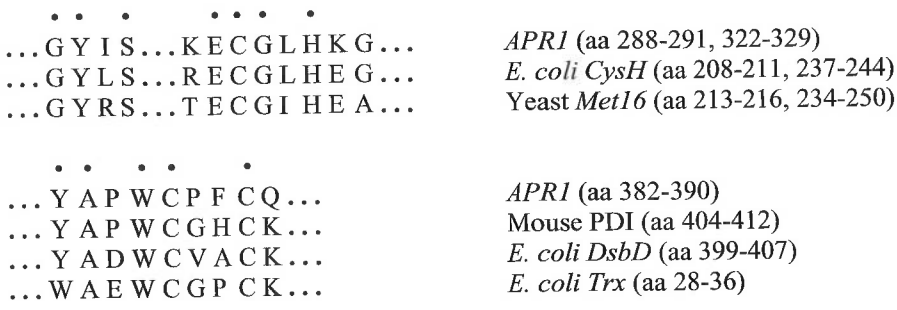



*Fig. 3.* Homology of *APR1* with PAPS reductases (top set) and disulphide exchange enzymes (bottom set). Identical or highly conserved amino acids in *APR1* are indicated with a solid dot. On the right of each sequence is its identity and the amino acid residues shown.

into a single functional polypeptide. How might these results relate to the proposed APS sulphotransferase of higher plants? In additional complementation tests we found that the *APR* clones are able to complement a *cysC*, APS kinase mutant (JM81A), but not a *cysD*, ATP sulphurylase mutant (JM221) of *E. coli*. This result suggests that the *APR* enzymes can use APS as a substrate. Interestingly, if the carboxyl terminus of *APR* functions in a manner analogous to thioredoxin, the enzyme would be expected to function as a thioredoxin-independent reductase capable of forming sulphite using any one of several reduced thiol compounds as a reductant. Interestingly, this is similar to the properties originally described for APS sulphotransferase.

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# ATP SULPHURYLASE EXPRESSION STUDIES IN YEAST AND IN PLANTS

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## Abstract

Sulphate starvation in both *Arabidopsis thaliana* and *Brassica napus* results in transiently increased abundance of the 1.9 kb ASA1 (*A. thaliana* ATP sulphurylase cDNA) hybridizing transcripts. The transcript is most abundant in plant aerial parts and shows the largest abundance increase in roots. Comparison of a yeast ATP sulphurylase mutant complemented with the yeast gene or ASA1 demonstrated differences in their ability to restore both enzyme activity and sulphate transport.

ATP-sulphurylase catalyses the thermodynamically unfavourable first step of sulphate assimilation by activating sulphate via an ATP-dependant reaction generating adenosine 5'-phosphosulphate. An *Arabidopsis thaliana* ATP sulphurylase cDNA (*ASA1*) has been cloned by functional complementation of a yeast ATP sulphurylase mutant[1]. The deduced encoded polypeptide (476 a a) contains a putative chloroplast signal peptide with a predicted cleavage site occurring before a region homologous to the N-terminal sequence obtained for spinach chloroplastic ATP sulphurylase[2]. Northern analysis of organs from *A. thaliana* and *Brassica napus* identified *ASA1* transcripts of 1.9 kb in all tissues examined (leaves, cotyledons, hypocotyls and roots), these being most abundant in aerial plant parts. An increase in *ASA1* transcripts relative abundance in response to sulphate deficiency was observed in both species with the largest relative increases occuring in roots. Studies of both relative ATP sulphurylase activity and transcript relative abundance in *B. napus* were carried out (Fig. 1) and indicate that initially the response to sulphate deprivation is transcriptionally regulated. Highest ATP sulphurylase activities were found in aerial tissues in agreement with previous studies and RNA abundance.

The yeast strain used for cloning *ASA1*, which has a point mutation in its ATP sulphurylase gene[3], is also defective in its sulphate transport capacity[4]. Comparison of this strain transformed with either the homologous gene (*MET3*) or heterologous cDNA (*ASA1*) showed that although both restored methionine heterotrophy and ATP sulphurylase activity (Table 1) only *MET3* complements the sulphate transport as demonstrated by $^{35}S$-sulphate influx measurements (Table 1).

This difference indicates an interaction between the yeast ATP sulphurylase and sulphate membrane transporter(s) leading us to propose channelling-like interactions between these two proteins. Such interactions could be envisaged as a mechanism for improving the thermodynamically unfavourable ATP-sulphurylase reaction. The poor restoration of sulphate transport by the plant ATP sulphurylase could be attributed to the non-cleavage of the chloroplast transit peptide affecting correct folding and function of the plant enzyme.

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*Table 1.* ATP sulphurylase activity and sulphate influx measurements in yeast. Two strains were used W303-1A (wild type) and CC371-4C (ATP sulphurylase mutant). *pM3-32* and p*ASA1* represent plasmids containing the yeast gene and the plant cDNA ATP sulphurylase clones, respectively. ATP sulphurylase activity was measured in the forward direction by molybdolysis[2]. Unidirectional [$^{35}$S]-sulphate influx measurements were performed for 3 min. in 0.1 mM $Na_2SO_4$ at 30°C after addition of 12.2 kBq to each tube. Protein content of the extracts were determined by the method of Lowry, using bovine serum albumin as a standard. Results are the mean of three independent experiments

| Yeast strains | Activity and Influx (nmol min$^{-1}$ mg$^{-1}$ protein) | |
|---|---|---|
| | ATP sulphurylase | [$^{35}$S]-sulphate influx |
| W303-1A | 538 | 46 |
| CC371-4C | 0 | 0 |
| CC371-4C + p*ASA1* | 356 | 8 |
| CC371-4C + p*M3-32* | 113 | 179 |

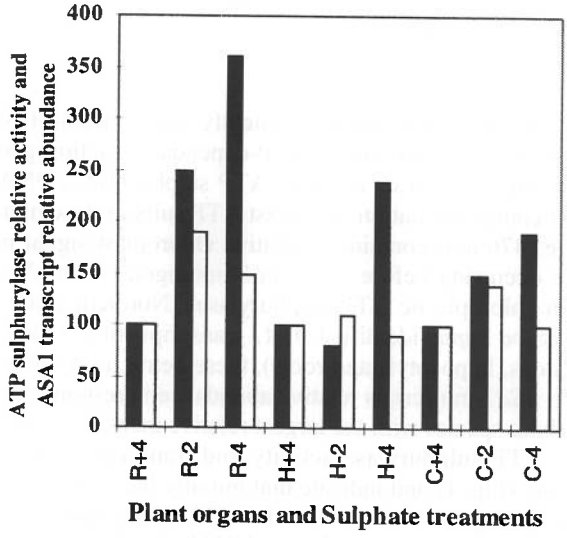


*Fig. 1.* ATP sulphurylase relative activities (■) and relative transcript abundance (□) in *Brassica napus*. Experiments were carried out on 8 day-old oilseed rape seedlings grown as already described[5]. Soluble proteins and total RNA were extracted from Roots (R), Hypocotyls (H) and Cotyledons (C) after growth on a complete nutrient solution containing 2 mM $MgSO_4$ for 4 days (+4), or in a medium in which sulphate has been replaced by 2 mM $MgCl_2$. for 2 days (-2) or 4 days (-4). ATP sulphurylase activity was measured in the forward direction by molybdolysis[2]. The relative abundance *ASA1* transcripts in each organ were determined by RNA slot-blot analyses, and standardized to actin hybridization. The variations of the *ASA1* transcript abundance and ATP-sulphurylase specific activities, in response to sulphate deficiency treatments (-2) and (-4), are expressed as a percentage of the sulphate non-limiting (+4) treatment. ATP sulphurylase specific activities for the sulphate non-limiting (+4) treatment were 104 (R), 169 (H) and 442 (C) nmol min$^{-1}$ mg$^{-1}$ protein.

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# SELENATE-RESISTANT MUTANTS OF *ARABIDOPSIS*

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## Abstract

Plants defective in sulphate uptake or reduction were sought by screening for *Arabidopsis* mutants resistant to the toxic sulphate analogue selenate. The selective media contained reduced sulphur in the form of methionine and enough selenate to prevent true leaf and root formation of wild-type plants. A total of eight plants whose progeny are clearly resistant to selenate were isolated from a screen of approximately 120,000 seeds. The selenate resistance in all cases segregates in genetic crosses as a single, nuclear, recessive trait, and the mutations form two complementation groups.

Many important questions about sulphur metabolism in plants remain unanswered[1]. Complex problems are often solved only through a combination of experimental approaches, due to the limitations inherent in each. However, just some of the available tools have been used to study sulphate assimilation in plants. Biochemical analysis, predominant in the past, continues to generate useful information but has not settled questions about which reactions observed *in vitro* occur *in vivo*[2]. Many of the genes of sulphate assimilation have recently been isolated from plants[3,4,5,6], and these molecular tools will undoubtedly prove to be extremely valuable. But a third line of attack that was the key to sorting out biochemical pathways in microorganisms has not been applied to sulphate uptake and reduction in plants. The missing approach is genetic – the isolation and analysis of mutants defective in the process of interest.

Mutants can be extremely useful in biochemical and molecular studies, but in addition they provide information that would be difficult to obtain in any other way. Specifically, they illustrate the biological relevance of a biochemical pathway by demonstrating the sometimes surprising consequences of a disruption. The importance of a particular metabolic intermediate can be tested directly in mutants unable to make that compound. Even the lack of an expected phenotype may be significant because it can disprove a hypothesis, or demonstrate genetic or functional redundancy of the affected enzyme. Individual mutants often have practical or experimental applications, especially if the affected gene has been isolated.

In order to identify mutants of the crucifer *Arabidopsis thaliana* defective in sulphate uptake or reduction, a genetic selection was devised. Selections, in which mutants survive conditions that kill wild-type plants, facilitate the rapid screening of large numbers of individuals. The selective agent used was the toxic sulphate analogue selenate ($SeO_4^{2-}$), which is taken up and reduced by the enzymes of sulphate assimilation, forming the seleno- analogues of cysteine and methionine[7]. Mutants impaired in sulphate import or reduction will be less sensitive to selenate because they will not produce as much of the seleno-amino acids. The feasibility of this approach has been demonstrated by the observation that yeast mutants disabled in sulphate permease, ATP sulphurylase, APS kinase, or PAPS reductase are all resistant to selenate[8].

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Because selenate is taken up by the sulphate transport system, the amount of selenate that enters the plant, and hence its apparent toxicity, will be influenced by the factors that regulate sulphate uptake. Therefore, the conditions under which selenate selections are performed must be a compromise between the need for sulphur to support growth and the down-regulation of sulphate assimilation by different sulphur-containing compounds[9]. High sulphate concentrations repress sulphate uptake[6] and also compete with selenate for import. Therefore, medium containing a small amount of sulphate (15 μM) was used in order to maximize selenate uptake. Mutants unable to import or reduce sulphate will require sulphur in a reduced form that does not enter via sulphate permease. It was empirically determined that methionine (100 μM) supports the growth of wild-type plants on low-sulphate medium, and that even though sulphate assimilation is also down-regulated by methionine, at this concentration the threshold of selenate toxicity is below 10 μM. Under these conditions, wild-type seeds germinate and the cotyledons expand but true leaves and roots never form. A screen for plants that could grow in the presence of selenate yielded eight mutants, at least five of which are independent, that consistently gave selenate-resistant progeny.

Characterization of the eight isolated mutants has just begun, but already demonstrates the success of the selection scheme. Genetic crosses clearly show that in all cases the selenate-resistance is due to a single, nuclear, recessive mutation, and that the mutations form two unlinked complementation groups (representative results are shown in Table 1). Crosses between G1 and each of the mutants not included in Table 1 gave only selenate-resistant plants in the $F_1$ and $F_2$ generations, indicating that each carries a mutation in the same gene. Planned experiments include measuring the rate of sulphate import in single and double mutants, mapping the mutations and identifying the biochemical defect caused by each, and testing the ability of cloned genes to complement the mutations. Additional selections for resistance to selenate and other agents will be undertaken with the goal of increasing the number of steps identified by mutation, and possibly isolating regulatory mutations. By combining a genetic approach with molecular and biochemical studies, we should gain a more complete understanding of how sulphate is assimilated by plants.

*Table 1.* Summary of genetic crosses with selenate-resistant mutants

| Cross | | Observed | | | Expected[a] | | |
|---|---|---|---|---|---|---|---|
| | | Sensitive | Resistant | Ratio | Sensitive | Resistant | Ratio |
| Wild-type X G1 | $F_1$ | 8 | 0 | 1:0 | 8 | 0 | 1:0 |
| | $F_2$ | 79 | 21 | 3.8:1 | 75 | 25 | 3:1 |
| Wild-type X B1 | $F_1$ | 8 | 0 | 1:0 | 8 | 0 | 1:0 |
| | $F_2$ | 74 | 25 | 3:1 | 74.25 | 24.75 | 3:1 |
| B1 X G1 | $F_1$ | 8 | 0 | 1:0 | 8 | 0 | 1:0 |
| | $F_2$ | 171 | 125 | 9.6:7 | 166.5 | 129.5 | 9:7 |

[a] Expected for single, nuclear, recessive mutations in unlinked genes

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# CYTOSOLIC *O*-ACETYLSERINE(THIOL)LYASE IS HIGHLY EXPRESSED IN TRICHOMES OF *ARABIDOPSIS*

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## Abstract

*Atcys-3A* from *Arabidopsis thaliana* encodes the cytosolic isoform of O-acetylserine(thiol)lyase. The transcript is present in different organs of mature plants. *In situ* hybridization studies reveal a high *Atcys-3A* expression localized to the cortex and xylem parenchyma in root, but the strongest *Atcys-3A* mRNA signal is found in trichomes of leaf and stem tissues. High levels of transcript is detected throughout the trichome developmental process. This cellular localization suggests that the cytosolic isoform of O-acetylserine(thiol)lyase may have a specialized function in trichomes.

Inorganic sulphate, the major source of sulphur for plants, is reduced and assimilated to L-cysteine, the principal starting metabolite for the synthesis of other sulphur-containing compounds such as methionine, glutathione and several secondary metabolites. The last step for L-cysteine biosynthesis from *O*-acetylserine and sulphide is catalyzed by *O*-acetylserine(thiol)lyase (EC 4.2.99.8). The presence of enzyme activity has been demonstrated in all three intracellular compartments involved in protein synthesis[1]. Several plant genes coding for the cytosolic, plastidic or mitochondrial isofoms of *O*-acetylserine (thiol)lyase have been isolated and characterized. In *A. thaliana* we have isolated a cDNA, *Atcys-3A*, encoding the cytosolic enzyme[2]. The *Atcys-3A* expression is activated by sulphur limitation, requiring carbon and nitrogen sources for maximal expression[2].

To characterize the tissue-specific expression of *O*-acetylserine(thiol)lyase in *Arabidopsis*, we have analyzed the *Atcys-3A* expression pattern in different organs of mature plants by northern blot analysis, and the spatial distribution of the transcript by *in situ* hybridization. Northern blot analysis shows a widely distributed expression of this gene, being most abundant in roots and 50% of the root level in leaves. This type of expression is expected for a cytosolic gene like *Atcys-3A*. The presence of *Atcys-3A* in all the organs of mature plants suggests that this gene product has an essential role to fulfil the thiol requirements of the plant metabolism.

*In situ* hybridization studies reveal a high *Atcys-3A* expression localized to the cortex and xylem parenchyma in roots (Fig. 1a,b). The transcript is undetectable in stems and is found at very low level in all leaf cell types. However, we find the strongest *Atcys-3A* mRNA signal in trichomes of either leaf or stem (Fig. 1c,d). Examination of different stages of trichome development[3], shows a high accumulation of transcript detectable throughout the developmental process. This pattern of expression suggests that the cytosolic isoform of *O*-acetylserine(thiol)lyase may have a specialized function in trichomes, besides its general role in primary sulphur assimilation. The capability of trichomes to have a higher cysteine biosynthetic activity may suggest a role of this cell type in the

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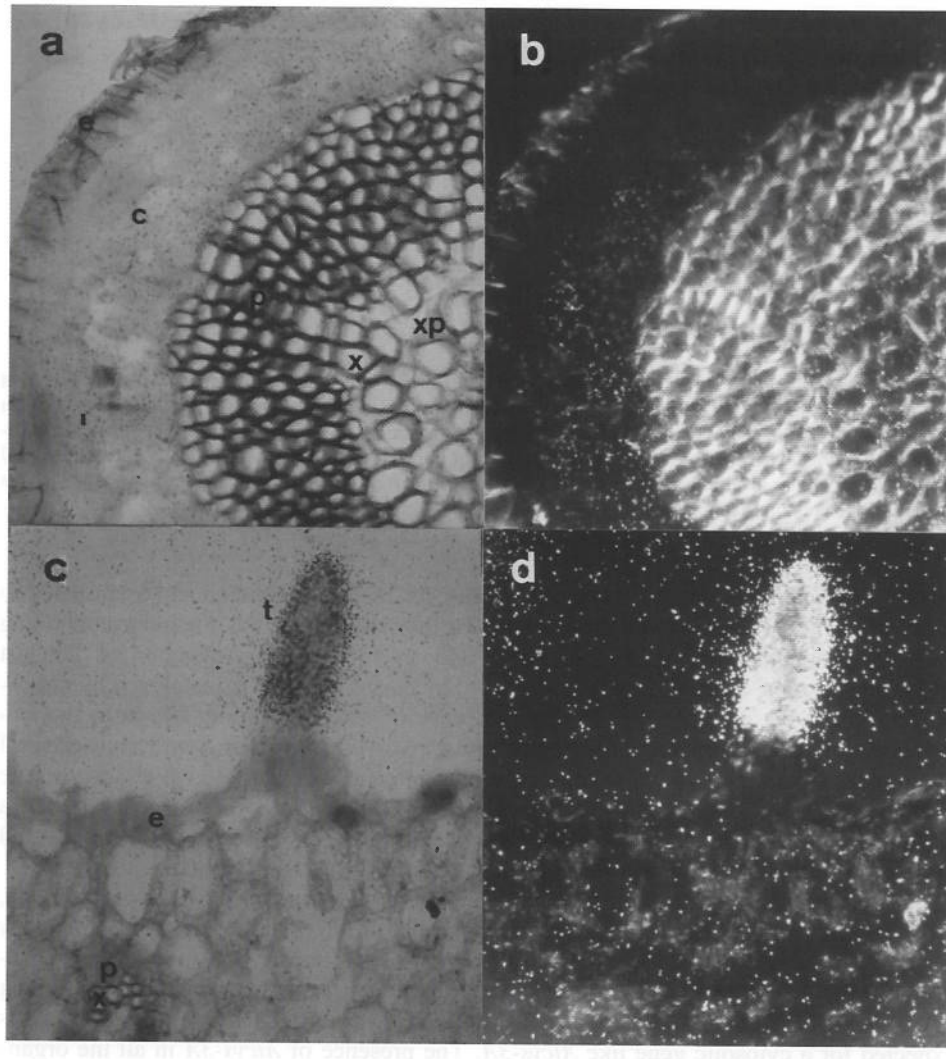


*Fig. 1.* Cellular localization of *Atcys-3A* transcript in *Arabidopsis*. (a) Bright and (b) dark-field micrographs of transverse section of root hybridized with antisense $^{35}$S-RNA. (c) Bright and (d) dark-field micrographs of transverse section of leaf hybridized with antisense $^{35}$S-RNA. *Arabidopsis* tissues were fixed, embedded in wax and sections were prepared on slides for *in situ* hybridization as previously described[7]. $^{35}$S-labelled RNA was synthesized using T7 RNA polymerase and *Atcys-3A* cDNA[2] as template in the presence of uridine [$^{35}$S] 5'-[α-thio] triphosphate. Hybridization was carried out in a moist chamber at 50°C overnight in 50% formamide. Slides were then washed, dehydrated and air dried. For autoradiography, slides were coated with Kodak NTB-2 emulsion and exposed 11 days at 4°C. After developing, the tissues were stained with toluidine blue. Photographs were taken with a Zeiss Microscope.
c, cortex; e, epidermis; p, phloem; t, trichome; x, xylem; xp, xylem parenchyma.

plant's response to some environmental stresses. A rapid oxidative burst is produced in plant cells in response to pathogenic attack[4], and the thiol compound glutathione plays a crucial role in the protection of plants against the harmful effects of active oxygen species[5]. Moreover, the heavy metal Cd is preferentially accumulated in trichomes of Indian

mustard[6] and this may be a possible metal detoxification mechanism. A major biosynthesis of cysteine would be required for complexing of metal ions by cysteine-rich proteins such as metallothioneins or phytochelatins.

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# MOLECULAR ANALYSIS OF THE CYSTEINE SYNTHASE COMPLEX FROM *ARABIDOPSIS THALIANA*

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## Abstract

Random mutagenesis of serine acetyltransferase (EC 2.3.1.30), a key component of the plant cysteine synthase complex, identified three amino acid residues essential for catalytic function. They are clustered in a junction between two structurally and functionally distinct domains, suggesting a close relationship between proper function of serine acetyltransferase and the formation of the cysteine synthase complex.

The final step of sulphate assimilation in plants consists in the formation of cysteine from sulphide and serine, catalyzed by the subsequent action of serine acetyltransferase (SAT) and O-acetylserine (thiol) lyase (OAS-TL). The work of Kredich and co-workers with *Salmonella typhimurium* first demonstrated the existence of a functional association of these two enzymes *in vitro* that was termed cysteine synthase (CS) complex[1]. A similar complex of SAT and OAS-TL has been demonstrated *in vitro* in protein extracts from several plant species[2,3]. The function of the CS complex has been assumed to be in metabolic channelling[4] or activation of SAT catalysis by OAS-TL[5].

Using the yeast two-hybrid system originally developed by Fields and Song[6], we have recently demonstrated two functionally independent protein-protein interaction domains within the SAT polypeptide *in vivo*[7]: a central region responsible for SAT/SAT association and a carboxy-terminal section providing SAT/OAS-TL interaction which correlated with two adjacent clusters of α-helical and β-pleated sheet stretches. The β-sheet area includes a transferase domain (amino acid 211-296 of SAT-A) that has been defined by structural, but not functional, comparison of bacterial acetyltransferases[8]. The carboxy-terminus of *A. thaliana* SAT thus might constitute a bifunctional domain responsible for catalysis and OAS-TL interaction with a decisive role in the assumed activation and/or channelling function of the CS complex.

The aim of this study was to locate the catalytic centre of SAT with respect to the OAS-TL protein interaction domain, using a putatively organelle localized isoform from *A. thaliana*[9]. We employed a heterologous genetic screening protocol to identify amino acid residues essential for catalytic activity that were created by random *in vivo* mutagenesis. SAT cDNA encoded on the expression plasmid pBS/DSAT1-6[9] was subjected to three overnight rounds of mutation in the *E. coli* mutator strain XL1-Red (Stratagene, La Jolla, USA) which is deficient in three DNA repair pathways and is supposed to create point mutations at every 1000-2000 basepairs of DNA. The library of mutated plasmids obtained was analyzed using the SAT deficient mutant *E. coli* strain EC1801 (*cysE*$^{-}_{i}$, generously provided by Dr. N. Kredich, Duke University, NC). Selection of cDNAs was performed via complementation of EC1801 on minimal medium without cysteine[9]: out of 918 transformants 73 clones were unable to grow and were further analyzed from replica

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plates. Plasmids from 69 null-mutant strains could be recovered and were tested in restriction analysis, leaving 17 candidates for point mutations. Out of the 17 clones only 3 remained without mutations in the promoter and polylinker region or stop codons in the SAT coding sequence. These clones were completely sequenced and revealed the following amino acid exchanges due to DNA point mutations: $Cys^{172}$ –> $Arg^{*172}$, $Ala^{202}$ –>$Val^{*202}$, $Ala^{220}$ –>$Pro^{*220}$. The three exchanges are sited within highly conserved amino acid motifs between the α-helix and β-sheet clusters, and right at the beginning of the putative transferase domain. $Arg^{*172}$ and $Pro^{*220}$ are likely to induce conformational changes, and although the $Ala^{202}$ –> $Val^{*202}$ transition is conservative, it significantly reduces the β-sheet probability according to secondary structure prediction analysis. The clustered position of the three mutations and their nature suggests that a region sensitive for proper function has been marked, rather than residues which are directly involved in catalysis. It is concluded that the structure of the SAT polypeptide in this area is not only important for protein-protein interaction but also for catalytic activity. These results will allow one further to characterize the molecular events inside the plant CS complex.

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# EXPRESSION ANALYSIS AND SUBCELLULAR LOCALIZATION OF CYSTEINE SYNTHASE ISOFORMS FROM *ARABIDOPSIS THALIANA*

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## Abstract

Three different isoforms of cysteine synthase from *Arabidopsis thaliana* were cloned and their respective expression pattern and subcellular localization determined by *in vitro* and *in vivo* uptake experiments. O-acetyl serine(thiol)lyase-*A (ASL-A)* encodes a cytosolic form, *ASL-B* a plastidic and *ASL-C* a mitochondrial isoform. *ASL-B* is regulated by light. While O-acetylserine stimulates transcription, cysteine represses transcription by feedback inhibition. Sulphate content of transgenic potato plants with reduced cysteine synthase activity due to antisense inhibition is not influenced.

Cysteine biosynthesis represents an essential step in the incorporation of reduced sulphur into organic sulphur in microorganisms and plants. Cysteine synthase (CSase; O-acetylserine(thiol)lyase) catalyses the formation of L-cysteine from O-acetylserine and inorganic and carrier-bound sulphide[1]. The enzyme consists of two identical subunits of about 35 kD with pyridoxal phosphate as cofactor.

Based on previous evidence[2, 6, 7], the presence of three isoforms of cysteine synthase has been suggested. Recently, cDNAs corresponding to three isoforms of CSase in spinach[9, 10, 11] and *A. thaliana*[4, 5] (Hesse *et al.* submitted) have been isolated. However, no data relating to the function, possible interaction of the various isoforms and regulation of cysteine biosynthesis in plant cells are available.

We cloned different cysteine synthase genes from *Arabidopsis thaliana* differing in transcript length and expression pattern. The cDNAs, having a length of 1278 bp (*ASL-A*), 1421 bp (*ASL-B*) and 1569 bp (*ASL-C*), encode for polypeptides of 324 (~34.6 kD), 392 (~41.8 kD) and 430 amino acids (~45.8 kD), respectively. Sequence comparisons of the predicted amino acid sequence with other plant cysteine synthase polypetide sequences[9, 10, 11] revealed a high degree of conservation for the mature part of all cysteine synthase isoforms even between different species.

The three different *ASL*-cDNAs were used as probes in RNA blot experiments to determine the expression pattern of cysteine synthase mRNA in leaves and roots of *A. thaliana*. Despite the high conservation of amino acid sequences each *ASL*-cDNA probe detects specifically under stringent conditions one transcript of 1.3, 1.5 and 1.6 kb in both tissues, suggesting that the *ASL*-cDNAs are full length. *ASL-A* transcripts were constitutively expressed in leaves and roots, while *ALS-B* is preferentially expressed in leaves. The *ASL-C* gene, which has not been decribed yet, is expressed in both tissues, but higher in roots when compared to leaves.

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**Localization of the *ASL-B* gene product.**

We could demonstrate that the *ASL-B* gene encodes for a chloroplastic isoform of cysteine synthase. *In vitro* translation products of *ASL-B* were taken up by isolated intact chloroplasts *in vitro*. These import experiments showed that the cysteine synthase precursor is proteolytically processed upon import into chloroplasts. The apparent molecular weights of the precursor (~42 kD) and imported mature (~36 kD) protein of the *Arabidopsis* peptide correspond to the predicted molecular weights. The 36 kD polypeptide was resistant to degradation by thermolysin indicating its localization within chloroplasts. The position of the processing site has been deduced from N-terminal amino acid sequencing of isolated protein from spinach[10, 11] and is conserved between different presumably plastidic localized isoforms.

**Subcellular localization of the *ASL-C* gene product**

Structural features of the *ASL-C* gene did not allow deduction of the subcellular localization of the protein. The predicted open reading frame encodes a protein of 45 kD. The deduced polypeptide of *ASL-C* shows a motif comparable to the cleavage site found for the chloroplastic transit peptide from spinach both in position and amino acid composition. However, compared to the plastidic isoform there is a novel N-terminal extension of 33 amino acids, supporting the assumption that the *ASL-C* product is localized in a subcellular compartment and not in the cytosol.

Two different approaches were applied to analyse the localization of the *ASL-C* gene product. *In vitro* uptake experiments with isolated mitochondria suggest a mitochondrial localization of the *ASL-C* gene product. A full-length *ASL-C* polypeptide was synthesized *in vitro* resulting in a translation product estimated to be 45 kD, which is in agreement with the calculated size of the *ASL-C* gene product. Uptake into isolated mitochondria of the *ASL-C in vitro* translation product leads to an appearence of a 37 kD polypeptide, which was resistant to degradation by proteinase K, indicating a mitochondrial localization. The addition of valinomycin, a potassium ionophore, prevents the import of translation products.

As a second approach, we determined *in vivo* localization of ASL-C by overexpression in transgenic tobacco plants. Two *ASL-C*/c-myc gene fusions were constructed and introduced into tobacco plants. Both constructs differ in length with respect to the putative presequence. The *ASL-C*/c-myc I construct contains the entire open reading frame of *ASL-C* whereas *ASL-C*/c-myc II has a shortened presequence starting at position +136, deleting the N-terminal extension.

Subcellular fractions of transgenic lines were tested in Western blot experiments of extracts from chloroplasts and mitochondrial fractions for the plants containing either *ASL-C*/c-myc I or II and for control plants transformed with the empty binary vector. The antibody raised against the c-myc epitope detected a fusion protein in crude extracts and enriched mitochondrial fractions in case of plants transgenic for ASL-C/c-myc I and as a contamination within the plastic fraction. In case of plants transgenic for ASL-C/c-myc II the antibody detected a fusion protein in crude extracts and fractions enriched for chloroplasts with the same apparent size that was detected in mitochondria. The ASL-C polypeptide is directed to mitochondria if the complete presequence is present, whereas the shortened presequence does not mediate import into mitochondria but rather into chloroplasts. FBPase and ANT antibodies were used to evaluate the quality of the subcellular fractions showing that the fractions were enriched for chloroplasts and mitochondria, respectively.

The reasons for (mis)targeting of *ALS-C*/c-myc II encoded protein into chloroplasts could be due to the fact that the truncated presequence of *ALS-C* starts with a motif of 16 amino acids which is conserved between the mitochondrial and chloroplastic isoform from *Arabidopsis* thus representing a functional transitpeptide. Furthermore, the mitochondrial isoform shows a conserved motif resembling the cleavage site of the transit peptide of chloroplastic isoforms[3,10]. In contrast, the comparison of the putative mitochondrial and the chloroplastic isoforms of spinach reveal greater differences than the *Arabidopsis* ones[10,11].

The high degree of homology found between the presequences of the mitochondrial and chloroplastic isoform allows speculations as to whether or not the mitochondrial form might be imported into chloroplasts *in vivo*, too. The results, so far, suggest that in plant cells a single presequence can interact functionally with the protein translocation systems of both chloroplasts and mitochondria, and raises the possibility that certain nuclear encoded proteins might be shared between these two organelles. It remains to be seen whether the enzyme can be imported by chloroplasts under specific conditions, such as those occuring under, e.g. methionine starvation.

## Analysis of cysteine synthase gene expression

To determine wether the transcript levels of cysteine synthase genes are regulated by abiotic and biotic factors, we performed Northern blot analyses of total RNA extracted from seedlings of *A. thaliana*. The plastidic cysteine synthase expression is regulated in a light dependent manner as it is described for nuclear encoded plastid localized enzymes. Expression of plastidic cysteine synthase decreased after 2 h in darkness and remained constant during a period of 24 h darkness while in light expression reached a maximum after 2 h compared with control plants and maintained constant even in a prolonged light period.

To test the possible influence of soluble metabolites on the expression of plastidic cysteine synthase seedlings were harvested after grown on AM-medium, or AM-medium supplemented with 5 mM O-acetylserine, nitrate, glutamate and cysteine and 5% sucrose, respectively. Nitrogen and carbon sources led to an increase in plastidic cysteine synthase steady state mRNA levels as compared to control while cysteine inhibited the expression, indicating modulation of cysteine synthase expression by metabolites, e.g. O-acetylserine as substrate stimulates and cysteine as product feedback inhibits expression.

Furthermore, we could detect a 6-fold increase (2-fold for each isoform) transcript accumulation by sulphur starvation but only a 2-fold increase in enzyme activity in crude extract. From these data we conclude that cysteine synthase is post-transcriptionally regulated.

*In planta* we tested whether cysteine synthase activity influences sulphate uptake. Analysis of transgenic potato plants with reduced enzyme activity due to expression of an antisense transcript of *ASL-A* and *ASL-B* isoforms from potato did neither show any phenotypic alterations nor altered sulphate contents in comparison to wild type plants.

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# CYSTEINE BIOSYNTHESIS IN HIGHER PLANTS: CLONING AND EXPRESSION OF THREE MEMBERS OF THE SERINE ACETYL-TRANSFERASE GENE FAMILY FROM *ARABIDOPSIS THALIANA*

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## Abstract

Three different cDNA clones, Sat-1, Sat-52 and Sat-53, encoding serine acetyltransferase (SAT) from *A. thaliana* have been isolated by functional complementation of the *E. coli cysE* mutant JM15 to prototrophy. Sequence analysis suggests that Sat-1 may encode a chloroplastic SAT isoform whilst the Sat-52 and Sat-53 encoded proteins may function in the cytoplasm. A hexapeptide repeat motif towards the C-terminus may be involved in the interaction of SAT with O-acetylserine (thiol) lyase. Preliminary northern analysis suggests that Sat-1 transcript levels in *A. thaliana* are highest in the root with moderate levels found in leaves. Much lower transcript levels are detected in stems, flowers and siliquas.

Serine acetyltransferase (EC 2.3.1.30) (SAT), a key enzyme in the biosynthesis of cysteine and of cysteine-derived metabolites in higher plants, catalyses the formation of *O*-acetylserine (*O*AS) from L-serine and acetyl-CoA. *O*AS is reported to be a limiting factor in cysteine biosynthesis and has also been implicated in cross-regulation of the converging pathways of sulphate and nitrate assimilation and in regulation of flux through the sulphate assimilation pathway[1,2]. Evidence from a number of plant species suggests the presence of isoforms of SAT distributed between cytoplasm[1,3], chloroplast[4] and mitochondrion[5,6]. As a first step towards analysing both the role of SAT in regulation, and the significance of its subcellular distribution, we have attempted to clone the SAT gene(s) by functional complementation of the *E. coli cysE* mutant JM15 to protrophy with an *A. thaliana* cDNA library in the expression vector λYES[7]. Analysis of over 100 complementing cDNA clones revealed the presence of three distinct groups represented by Sat-1 (8; GenBank Accession Number: U22964), Sat-52 (J.R.Howarth, M.A.Roberts & J. L. Wray, unpublished; GenBank Accession Number: U30298) and Sat-53 (equivalent to Sat-5 of Ruffet et al. (1995)[6] ; GenBank Accession Number: Z34888). pSAT1 and pSAT52 were able to restore SAT activity, and prototrophy, to the *cysE* mutant (pSAT53 was not tested).

Dot-plot analysis of the three SAT deduced amino acid sequences reveals the presence of a hexapeptide repeat motif, $([IVL]GXXXX)_5[IVL]$, towards the C-terminus that is characteristic of a range of transferase enzymes (Figure 1). We speculate that secondary structures formed by these repeats[9] may be involved in the interaction of SAT with *O*-acetylserine (thiol) lyase[4]. Although functional assays will be required to identify

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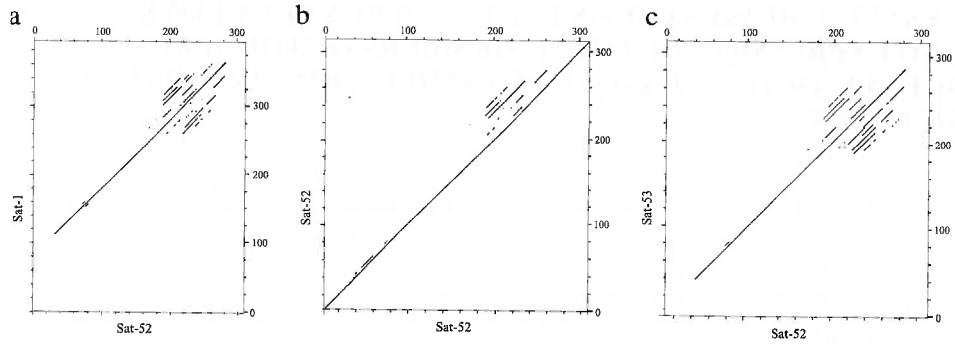


*Fig. 1.* Dot-plots of the deduced SAT-52 amino acid sequence compared with a) SAT-1, b) itself and c) SAT-53. Diagonals offset from the main self-identity line reveal internal repeated amino acid sequences. Comparisons were made using GCG Dotplot and Compare programs with a window of 60 and a stringency of 25.

unequivocally the subcellular location of the encoded proteins preliminary sequence analysis of the N-terminus of the deduced SAT proteins suggests that SAT-1 is targeted to the chloroplast whilst SAT-52 and SAT-53 may function in the cytoplasm.

Gene-specific probes have been constructed and are being used to study expression of these members of this small gene family. Northern analysis of RNA extracted from roots, leaves, stems, flowers and siliquas of compost-grown plants indicates that the Sat-1 transcript levels are highest in the root with moderate amounts also detected in the leaves. Much lower transcript levels are detected in stems, flowers and siliquas. These preliminary results suggest that the SAT-1 protein may also be localised in root plastids.

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# CYSTEINE DESULPHYDRASE OF *SPINACIA OLERACEA* L. MAY CATALYZE THE SYNTHESIS OF CYSTEINE

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## Abstract

In crude extracts of spinach cotyledons cysteine could be synthesized from $Na_2S$ and ß-chloro-L-alanine. Apparent $K_m$ values were 0.24 and 1.4 mM for sulphide and ß-chloro-L-alanine, respectively.

Under normal conditions, cysteine content in plants is maintained at very low levels[1] and it has been proposed that cysteine desulphydrase plays an active role therein[2]. This enzyme catalyzes the degradation of cysteine, resulting in the formation of sulphide, pyruvate and $NH_4^+$. In *Aerobacter aerogenes*, however, cysteine desulphydrase is able to synthesize cysteine from sulphide and, as an artificial substrate, β-chloro-L-alanine[3]. Moreover, Schütz *et al.*[4] presented circumstantial evidence that in extracts of pumpkin leaves cysteine desulphydrase might be able to synthesize cysteine from atmospheric $H_2S$, in its reversed reaction. For L-cysteine degradation Schütz[5] observed an apparent $K_m$ for L-cysteine of 4.3 mM, which is two orders of magnitude higher than the cysteine concentration usually observed in plant tissue.

7-10 days old spinach (*Spinacia oleracea* L. cv. Subito) cotyledons were homogenized in ice-cold 25 mM Tris/HCl pH 8.0. The homogenate was filtered through one layer of Miracloth. The reaction was carried out in closed 1.5 ml Eppendorf reaction tubes. The standard reaction mixture contained 200 µl plant extract, 100 µl 0.5 M potassium phosphate buffer, pH 7.0, 50 µl 4 mM pyridoxal 5'-phosphate and 50 µl 0.25 M β-chloro-L-alanine. The reaction was started by the addition of 50 µl 0.1 M $Na_2S$. The pH of the reaction was 9.4. Incubation time was 20 minutes at 23°C. The reaction was stopped by the addition of 1 ml of Gaitondes reagent[6]. The cysteine formed was determined colorimetrically as described by Gaitonde[6].

The rate of cysteine formation from sulphide and β-chloro-L-alanine was strongly temperature dependent (Fig. 1a). The $Q_{10}$ was 2 indicating an enzyme mediated reaction. Up to 54°C there was no temperature optimum for the reaction. The reaction showed a pH optimum at pH 12 or higher (Fig. 1b). At pH 5 or lower, there was no detectable formation of cysteine. The pH dependency of the reaction coincided more or less with the dissociation curve of $H_2S$ to $SH^-$. This may indicate a preference for $SH^-$ rather than $H_2S$ or $S^{2-}$, as a substrate for the cysteine synthesis. The rates of cysteine synthesis showed a rather narrow optimum around 1 mM sulphide (Fig. 1c). The apparent $K_m$ for sulphide was 0.24 mM, which is in the same range as reported for cysteine synthase[7], and one order of magnitude lower than the $K_m$ value for L-cysteine in its degradation reaction by desulphydrase[5]. The rate of cysteine synthesis showed a narrow optimum around 7 mM β-chloro-L-alanine and the apparent $K_m$ was 1.4 mM (Fig. 1d). The maximum specific activity of

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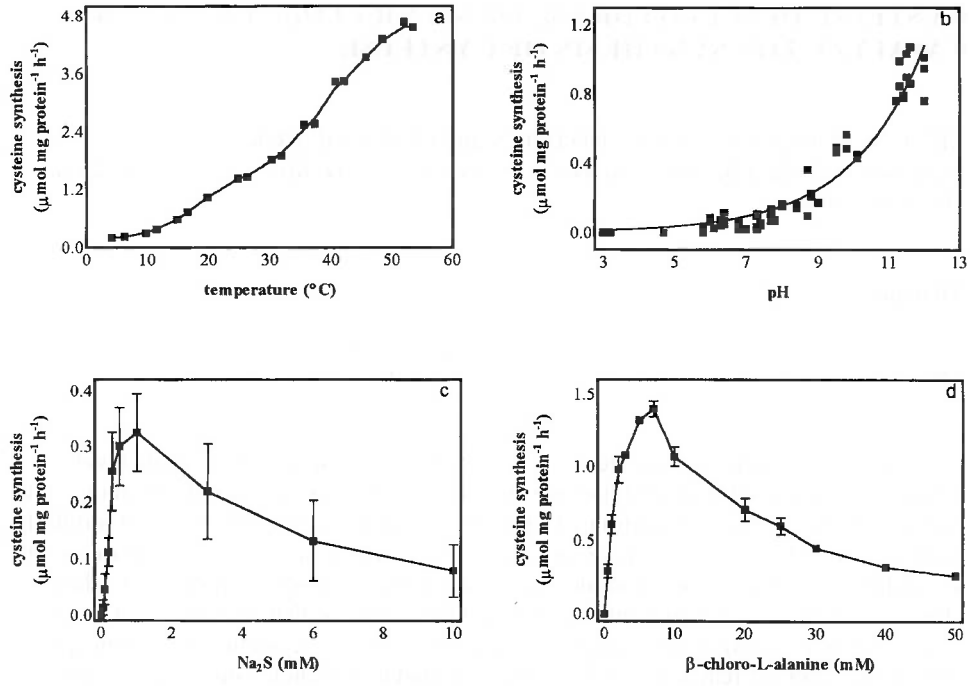


*Fig. 1.* Synthesis of cysteine catalyzed by spinach cotyledon extracts with $Na_2S$ and β-chloro-L-alanine as substrates. Dependency on temperature (a), pH (b), and on the concentations of $Na_2S$ (c) and β-chloro-L-alanine (d; measured at 1 mM $Na_2S$). The data (c, d) represent the mean of three independent measurements (± SD).

cysteine synthesis was 1.4 µmol mg protein$^{-1}$ h$^{-1}$. The maximum level of cysteine synthesis on a fresh weight basis was 6 µmol g FW$^{-1}$ h$^{-1}$, which was much lower than the extractable levels of cysteine synthase found in herbaceous plants (4800 µmol g FW$^{-1}$ h$^{-1}$)[8]. Apart from β-chloro-L-alanine, *O*-acetyl-L-serine, N-acetyl-DL-serine, L-alanine, L-phenylalanine, L-threonine and L-serine were tested as substrates for the cysteine synthesis under the incubation conditions described. None of them exhibited cysteine formation. The physiological significance of the described reaction of cysteine synthesis and its "natural" substrate, instead of β-chloro-L-alanine, needs to be determined and further established.

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# MOLECULAR CHARACTERIZATION AND REGULATION OF CYSTEINE SYNTHASE AND SERINE ACETYLTRANSFERASE FROM PLANTS

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## Abstract

The cDNAs encoding three isoforms of cysteine synthase were isolated and characterized from spinach. These isoforms are localized in cytosol, chloroplasts and mitochondria. The expression of genes for the isoforms are differentially regulated by sulphate and nitrate nutrition. We isolated the cDNAs encoding serine acetyltransferase from spinach and watermelon and investigated their expression pattern. Transgenic plants expressing the cDNA of cysteine synthase were made to study the role of cysteine synthase in cysteine biosynthesis. The mechanism for regulation of cysteine biosynthesis is discussed.

The cysteine biosynthetic pathway is responsible for assimilation of sulphur in plants[1]. Cysteine synthase (CSase) [O-acetylserine(thiol)-lyase] catalyzes the formation of L-cysteine from O-acetyl-L-serine (OAS) and hydrogen sulphide. OAS is supplied by serine acetyltransferase (SATase) from acetyl-CoA and serine. CSase and SATase in conjunction are assumed to play regulatory roles in cysteine biosynthesis in plant cells[2].

The enzymes participating in the reduction of sulphate to sulphide are believed to be almost exclusively localized in chloroplasts[3]. In fact, the cDNAs encoding all enzymes involved in the non-bound pathway of sulphate reduction have been isolated from higher plant cells [other chapters in this book]. Physiological evidence indicates that CSase in spinach leaves is localized in cytosol, chloroplasts and mitochondria[4]. Recently, we have isolated nucleus-encoding cDNAs for isoforms from the different subcellular locations; CSaseA (*CysA*) in the cytosol[5,6], CSaseB (*CysB*) in chloroplasts[7], and CSaseC (*CysC*) in mitochondria[8]. The amino acid sequences of the three isoforms exhibit 60~75% homology. The pyridoxal-phosphate cofactor binding lysine site, which is identified by extensive site-directed mutagenesis of CSaseA protein, was highly conserved[9]. The extension of N-terminal sequences of the three isoforms differed to direct the transport of the catalytic proteins to different sub-cellular compartments. CSaseA has no N-terminal extra sequence; whereas CSaseB and CSaseC have the transit peptide (TP) sequences for transportation to organelles. To confirm the identity of these isoforms for organelle localization, the DNA sequences encoding these TPs were fused to the reporter β-glucuronidase (*gus*) gene, and these chimeric genes were used for transformation of tobacco plants. The experiments on subcellular localization of Gus protein clearly indicated the transportation of Gus to chloroplasts and mitochondria directed by CSaseB-TP and CSaseC-TP, respectively[10].

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SATase activity is also detected in cytosol, chloroplasts and mitochondria as well as CSase. The highest activity was localized in mitochondria of pea[11]. Of several cDNA clones isolated from plants, the clone from watermelon was proven to encode a cytosolic form by Western blot analysis[12]. The clone from spinach which possessed an N-terminal extension of ~50 amino acids is presumed, from the data of peptide structure and expression pattern, to encode the mitochondrial form.

Taking into account all the data available so far, it is evident that the post-sulphide reactions catalyzed by SATase and CSase take place in cytosol, chloroplasts and mitochondria, where *de novo* protein biosynthesis occurs. This may imply that cysteine cannot be transported across organelle membranes. Since the concentration of cysteine in cells is strictly controlled at a low level (~10 nmole/g fresh weight), it is probably difficult for cysteine to move by a non-specific amino acid transporter. If there is no cysteine-specific transporter in the cells, each organelle needs to supply the cysteine necessary for protein biosynthesis inside the organelle.

Some insight into the regulation of cysteine biosynthesis has been obtained at the enzyme level by using recombinant enzymes, and at the transcriptional level by using cDNAs as probes. SATase and CSase forms a multienzyme complex *in vitro* and presumably also *in vivo*, suggesting efficient metabolic flow from serine to cysteine preventing the diffusion of intermediary OAS[12]. The SATase activity of the recombinant enzyme from watermelon was inhibited by L-cysteine, a final product of the biosynthetic pathway, in a typical non-competitive manner ($K_i$=3.7 μM and 5.6 μM for L-serine and acetyl-CoA, respectively). This inhibitory effect was specific to L-cysteine ($IC_{50}$ = 2.9 μM), only weak activities being detected with D-cysteine and N-acetyl-L-cysteine. L-Cystine, DL-homocysteine, L-methionine, glutathione and $SO_4^{2-}$ ions showed neither an inhibitory nor a stimulatory effect up to 1 mM. β-Pyrazolealanine is produced from OAS and pyrazole by CSase in watermelon. However, no inhibitory effect was detected on β-pyrazolealanine which is also assumed to be an end product of this biosynthetic pathway. These results indicated that the feedback inhibition of SATase plays an important regulatory role in the cysteine biosynthetic pathway but does not operate in β-pyrazolealanine production. This difference in regulation can explain the 1000-fold difference in the cellular concentrations of cysteine (~10 nmole/g fresh weight) and β-pyrazolealanine (~10 μmole/g fresh weight) (Fig. 1).

In terms of transcriptional control of CSase genes, *CysA* and *CysC* were expressed in green and non-green parts of plants; whereas *CysB* was expressed primarily in green parts[7,8]. When starved of sulphur and nitrogen, the mRNA accumulations of *CysA* and *CysB* in spinach cell culture were enhanced by a factor of about 1.5-fold in 24hr, but no remarkable change was observed (Table 1). However, the amount of *CysC* transcript increased up to 5-fold under nitrogen deficiency and nitrogen/sulphur double deficiency conditions. The change of SATase mRNA level showed the same trend, i.e. a slight enhancement, as that of CSaseA and CSaseB under sulphur deficient conditions in spinach cell culture and in watermelon seedlings. However, the addition of pyrazole stimulated the mRNA accumulation of SATase in watermelon seedlings which produce β-pyrazolealanine.

The nucleotide sequence of the 5'-flanking region (~1 kb) of the *Sat* gene of watermelon was determined, and the transcriptional start point was determined by the primer extension method. Several interesting features such as direct and inverted repeats and palindromic sequences were found in addition to general promoter motifs like TATA box and CAAT box. Two characteristic recognition motifs of SEF4 factor[13], which were found in soybean and identified as a trans factor responding to sulphur deficiency, were identified in the promoter sequence of the *Sat* gene.

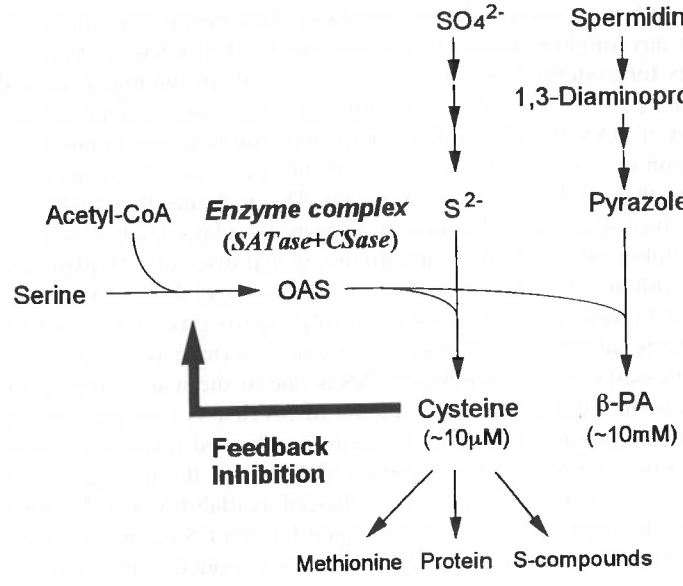


*Fig. 1.* Regulation of biosynthesis of cysteine and β-pyrazolealanine.

We have investigated the modulation of cysteine formation responding to biosynthetic precursors in transgenic tobacco (*Nicotiana tabacum*) expressing a foreign cysteine synthase[14]. Three T-DNA vectors carrying a spinach cytosolic CSaseA cDNA were constructed: pCSK3F, cDNA driven by CaMV 35S promoter with a sense orientation; pCSK3R, cDNA driven by CaMV 35S promoter with an antisense orientation; pCSK4F, cDNA fused with the sequence for the chloroplast-targeting transit peptide of pea ribulose 1,5-biphosphate carboxylase small subunit driven by CaMV 35S promoter with a sense orientation. These chimeric genes were transferred into tobacco.

The spinach CSaseA expressed in tobacco was accumulated as catalytically active protein in the cytosol of 3F transformants and in chloroplastic stroma of 4F transformants. CSase activities in chloroplasts of pCSK4F transformants were several to ten fold higher than those of control and pCSK3F plants. In spite of enhanced chloroplastic CSase activity in 4F plants, cellular and chloroplastic contents of cysteine and glutathione were not

*Table 1.* Transcriptional control of gene expression of cysteine synthase (CSase) and serine acetyltransferase (SATase). No, no significant induction was observed; n.d., not determined

| Protein | Gene | Source plant | Subcellular fraction | Induction on mRNA accumulation (% of control)[a] | | |
|---|---|---|---|---|---|---|
| | | | | Minus S | Minus N | Plus pyrazole |
| CSase | *CysA* | Spinach | Cytosol | ~150% | No | n.d. |
| | *CysB* | | Chloroplasts | ~150% | No | n.d. |
| | *CysC* | | Mitochondria | No | 500% | n.d. |
| SATase | *SatA* | Watermelon | Cytosol | ~150% | No | ~200% |
| | *SatC* | Spinach | Mitochondria? | ~150% | No | n.d. |

[a] Control: S and N sufficient = 100%

significantly changed in a normal growth condition. This means that, under the normal condition without any sulphur stress, the endogenous level of CSase is nearly sufficient for the normal flux for cysteine biosynthesis and is not a major limiting step for this pathway. In chloroplasts, OAS was a dominant limiting factor for cysteine formation, and combined addition of OAS and the sulphur compounds, sulphite and sulphide, resulted in enhanced production of cysteine. These enhancements were more pronounced in chloroplasts of a 4F plant than in those of a control plant. This indicates that, under the sulphur stress conditions, over-accumulated CSase in chloroplasts plays a substantial role in fixing over-loaded sulphur into cysteine. Furthermore, in leaf discs of a 4F plant, excess sulphite resulted in enhanced cysteine production. These leaf discs over-accumulating CSase in chloroplasts were partially resistant to sulphite toxicity. A working model can be proposed for the regulatory mechanism of cysteine biosynthesis in chloroplasts accumulating over-expressed cysteine synthase. OAS is one of the major limiting factors for cysteine biosynthesis in chloroplasts. The amount of SATase is very low compared with that of CSase and the maximal cysteine synthesis is achieved under the condition of a large excess of CSase to SATase. Over-expressed CSase in the transgenic plants may require more amounts of SATase leading to enhanced availability of OAS for maximal cysteine formation. In conclusion, over-accumulated foreign CSase in chloroplasts could modulate biosynthetic flow of cysteine responding to over-loaded sulphur stress.

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# PURIFICATION OF TWO TISSUE-SPECIFIC ISOFORMS OF *O*-ACETYLSERINE (THIOL) LYASE FROM SPINACH (*SPINACIA MEDANIA* L.) AND INFLUENCE BY SULPHUR NUTRITION

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## Abstract

O-Acetylserine (thiol) lyase activity (OAS-TL) was demonstrated in spinach leaf and root tissue with the major part in the leaf tissue (74% of total activity). Following a 6-day sulphur starvation period, the OAS-TL activity in the leaves was decreased to 50% of the total activity in the plant, whereas the total activity in the plant remained constant. Compartmental analyses of leaf and root tissues revealed the presence of different isoforms of OAS-TL. In leaves, the chloroplastic isoform dominated clearly over the cytosolic form. Following sulphur starvation, chloroplastic and cytosolic OAS-TL activity showed a more even distribution. The purified chloroplast enzyme had a subunit size of 36 kD and a strong cross-reactivity with an anti-chloroplast OAS-TL antibody. In root tissue, only one major form of the enzyme was present in the cytosolic fraction. The purified root enzyme had a subunit size of 34 kD. Cross-reactivity with the antibody to the chloroplast enzyme was weak.

*O*-acetylserine (thiol) lyase [OAS-TL; EC 4.2.99.8] catalyses the synthesis of L-cysteine from the precursors *O*-acetylserine and sulphide. Sulphur assimilation takes place predominantly in the chloroplasts but has been demonstrated in root tissue also[3]. OAS-TL was shown to be present in chloroplasts, cytosol and mitochondria of spinach leaf tissue[9,5,6,10,11] indicating the capability of these compartments for cysteine synthesis.

In the present study, OAS-TL activity was compared in spinach leaf and root tissue, with the majority of the enzyme activity found in the leaf tissue (74%; Table 1), supporting the idea that sulphur assimilation occurs predominantly in the leaves. Following the separation of different subcellular compartments, chloroplasts appeared to contain the major part of the leaf OAS-TL activity, but some activity was also found in the cytosolic fraction (Table 1). The chloroplast/plastid yield was between 15 and 22% of the original starting material, as determined by the plastid marker enzyme nitrite reductase (NiR) (Table 1). Contamination of the chloroplast/plastid fraction by other organelles or cytoplasm was less than 1% (data not shown). The purified chloroplast OAS-TL had a subunit size of 36 kD as determined by SDS-PAGE (Figure 1, lane 2) and cross-reacted strongly with an anti-chloroplast OAS-TL antibody from spinach.

In root extracts, the major part of the OAS-TL activity was located in the non-plastid fraction (Table 1). This coincides well with results from spinach roots[7] and pea roots[4] were predominant activities of OAS-TL were also observed in the cytosolic fraction. The purified root OAS-TL, very likely representing the cytosolic isoform of the enzyme, had

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*Table 1.* Typical distribution of OAS-TL activity in spinach plants between leaves and roots, and between plastid and non-plastid fractions. Chloroplasts were purified from hydroponically-grown spinach leaf tissue following the method of Bartlett et al.[1]. Root plastids were prepared from the above plants based on the method of Bowsher et al.[2]. Chloroplast and root plastid yields were determined using nitrite reductase (NiR) as a marker enzyme. The data shown are taken from a single representative experiment. All values are given as nmol cysteine $min^{-1}$ g $fw^{-1}$. Values in ( ) are percentage of total OAS-TL activity.

| | | *O*-acetylserine (thiol) lyase activity [nmol cysteine $min^{-1}$ g $fw^{-1}$] | |
|---|---|---|---|
| | | Control | Sulphur starved |
| Leaf | crude extract | 209 (74%) | 110 (43%) |
| | chloroplast fraction | 29 | 6 |
| | non-plastid fraction | 6 | 8 |
| | chloroplast yield | 22% | 15% |
| Root | crude extract | 73 (26%) | 149 (57%) |
| | plastid fraction | 4 | 5 |
| | non-plastid fraction | 54 | 61 |
| | plastid yield | 19% | 17% |
| Leaf and Root | | 282 (100%) | 259 (100%) |

a subunit size of 34 kD, slightly smaller than that of the chloroplastic OAS-TL (Figure 1, lane 3). When blotted against the anti-chloroplast OAS-TL antibody, a weak cross-reactivity was detected for the cytosolic OAS-TL (Figure 1, lane 5). Furthermore, a contamination of the preparation with, presumably, proplastidic OAS-TL in low abundance became obvious. Non-denaturing gels (Figure 2) supported the above assumption of a major cytosolic OAS-TL isoform in roots and a different isoform in chloroplasts. In crude leaf extracts, two bands of different mobility were observed (Figure 2, lane 1), one

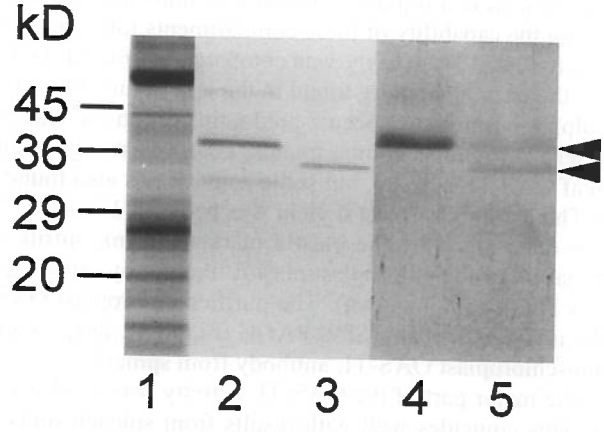


*Fig. 1.* SDS-PAGE of purified chloroplastic and root OAS-TL. Spinach plants were grown for 4-6 weeks in pots containing Levington M3 mix (Fision, UK) or in a liquid Letcombe nutrient solution[8]. Where required, hydroponically-grown plants were sulphur-starved for 6 days prior to harvest. OAS-TL isozymes were purified following a modified protocol of Droux et al.[6]. 1: crude leaf extract; 2: purified chloroplast OAS-TL; 3: purified root OAS-TL; 4 and 5: Western blots with anti-chloroplast OAS-TL antibody; 4: purified chloroplast OAS-TL and 5: purified root OAS-TL.

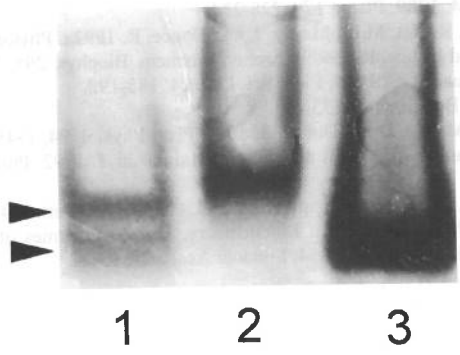


*Fig. 2.* Non-denaturing PAGE of spinach leaf, chloroplast and root extracts. Crude leaf (1; 80 µg protein) and root (3; 80 µg protein) extracts and a chloroplast fraction (2; 20 µg protein) were run on a 5% (w/v) non-denaturing PAGE. Gels were stained for enzyme activity following a method described by Schmidt[12].

of which showed similar mobility to the band of activity detected in chloroplasts (Figure 2, lane 2). The other band presumably represented the cytosolic form of the OAS-TL as seen by comparison with the crude root extract (Figure 2, lane 3), where the major portion of the OAS-TL was shown to be in the cytosolic compartment.

Following a 6-day sulphur starvation period, the whole plant OAS-TL remained fairly constant (Table 1), whereas the enzyme activity in the leaves was decreased by a factor of two and the root OAS-TL activity was increased by a similar factor (Table 1). The enzyme distribution between plastid and cytosolic fraction remained unchanged in roots. Therefore the observed increase in OAS-TL activity in the roots must be attributed to an enhanced expression of the cytosolic OAS-TL under sulphur starvation, possibly indicating a higher demand for reduced sulphur in the root. In leaves, however, a pronounced decrease in chloroplastic OAS-TL could be observed under these conditions, resulting in an even distribution of OAS-TL between chloroplast and cytosolic fraction. The data presented indicate that activities of OAS-TL isoforms are being regulated by the sulphur supply of the plant. Whether the actual expression of the isoforms is influenced in the same way remains to be shown.

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# REGULATION OF GLUTATHIONE SYNTHESIS IN TRANSFORMED POPLARS

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## Abstract

Transformation of poplars to overexpress γ-glutamylcysteine synthetase (γ-ECS) brought about marked increases in foliar quantities of γ-glutamylcysteine (γ-EC) and glutathione (GSH). The relative increases in these two thiols were light-dependent: the content of γ-EC was high in darkened leaves and low in illuminated leaves, whereas GSH content varied inversely.

The factors which regulate the foliar content of glutathione have not been unequivocally established. Among the likely candidates are the availability of cysteine and the *in vivo* activities of the two enzymes responsible for the synthesis of glutathione from constituent amino-acids (γ-glutamylcysteine synthetase (γ-ECS: EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3)). In order to assess the influence of the latter, we have used the corresponding bacterial genes to transform poplars to overexpress these enzymes. We have previously shown that strong overexpression of glutathione synthetase in poplar (up to 100-fold increase in extractable activity) had no effect on foliar glutathione levels, suggesting that the endogenous activity of this enzyme does not limit the rate of glutathione synthesis[1,2]. In contrast, Table 1 demonstrates that the foliar glutathione content is markedly enhanced (approximately 3-fold) in poplars which express the *E.coli* gene for γ-ECS (transformant gsh282d). Foliar γ-glutamylcysteine (γ-EC) content is also approximately 10 to 15-fold greater than in untransformed controls. The increased glutathione found in this transformant indicates that the endogenous activity of γ-ECS exerts significant control over the rate of γ-EC synthesis and thereby imposes a limitation on the amount of glutathione in the leaf. Moreover, it may be inferred that, under conditions existing in the transformants, increased synthesis of γ-EC (and consequently glutathione) is not prevented by shortage of cysteine, since cysteine contents do not decrease in the transformants (Table 1).

Under conditions in which the capacity for γ-EC synthesis was increased by fumigation of plants with $H_2S$, it was found that γ-EC, normally present in negligible quantities, increased markedly in darkened spinach leaves[3]. Figure 1 shows that a similar phenomenon may be observed in transformed poplars with high γ-ECS activity (upper figure, closed circles). In these plants, foliar γ-EC is highest in the dark and declines on illumination to 10-15% of dark values. This light-induced decrease in γ-EC is mirrored, in gsh282d poplars, by a corresponding increase in the glutathione level, which doubles

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*Table I*[a]. Effect of γ-glutamylcysteine synthetase overexpression on foliar glutathione and γ-glutamylcysteine contents in poplar. The *E.coli* gene for γ-ECS was introduced into poplar by *Agrobacterium* and lines with highest activity of γ-ECS were chosen for analysis. In line gsh282d, activity was 8.67±1.56 nmol $mg^{-1}$ prot. $min^{-1}$. We are unable to detect γ-ECS activity in untransformed poplars; by comparison with literature data, we estimate a 24-fold increase in the line gsh282d. Thiols were determined fluorometrically following derivatisation with monobromobimane and separation by HPLC, in 3 identical experiments[2]. Values represent the mean of 4 extractions ± sd, from the 7th mature leaf

| Thiol | Experiment | Thiol contents/ | | Increased content in transformant |
|---|---|---|---|---|
| | | In untransformed poplar | In transformant gsh282d | |
| Total glutathione | 1 | 255 ± 26 | 751 ± 4* | 3.0 |
| | 2 | 173 ± 12 | 482 ± 51* | 2.8 |
| | 3 | 258 ± 13 | 1071 ± 25* | 4.2 |
| Oxidised glutathione | 1 | 10.8 ± 0.4 | 30.0 ± 3.1* | 2.8 |
| | 2 | 11.6 ± 0.8 | 45.7 ± 1.2* | 3.9 |
| | 3 | 12.2 ± 0.8 | 41.1 ± 4.6* | 3.4 |
| Glutamylcysteine | 1 | 9.2 ± 1.1 | 99.7 ± 12.8* | 10.8 |
| | 2 | 22.5 ± 3.1 | 265.4 ± 8.0* | 11.8 |
| | 3 | 7.6 ± 0.6 | 115.0 ± 4.6* | 15.1 |
| Cysteine | 1 | 12.6 ± 0.3 | 18.9 ± 2.1 | 1.5 |
| | 2 | 6.8 ± 0.3 | 17.5 ± 2.6 | 2.6 |
| | 3 | 7.4 ± 0.7 | 9.6 ± 1.0 | 1.3 |

*P<0.01 Student t-test

[a] Work carried out in conjunction with Dr. Michael Strohm and Prof. Heinz Rennenberg, Institut für Forstbotanik und Baumphysiologie, Albert-Ludwigs-Universität, Freiburg, Germany.

under illumination (closed circles, lower figure). More than half of this increase can be accounted for by utilisation of γ-EC already present in the dark (compare closed circles, upper and lower figures). These complementary changes in γ-EC and glutathione contents are much less dramatic in both untransformed poplars and in poplars transformed to express a bacterial gene for glutathione reductase (120-fold increase in extractable activity; triangles). In these plants, although γ-EC does become measurable in the dark, the amounts which accumulate never exceed 10% of the dark values observable in the gsh282d poplars, which possess much greater γ-ECS activity (upper figure, compare closed circles with open circles and triangles). This indicates that the diurnal changes shown here may only become manifest when the capacity for synthesis of γ-EC is greatly enhanced: work is in progress to determine whether the increased γ-EC in the dark is owing to a shortage of glycine in the absence of photorespiration[3,4].

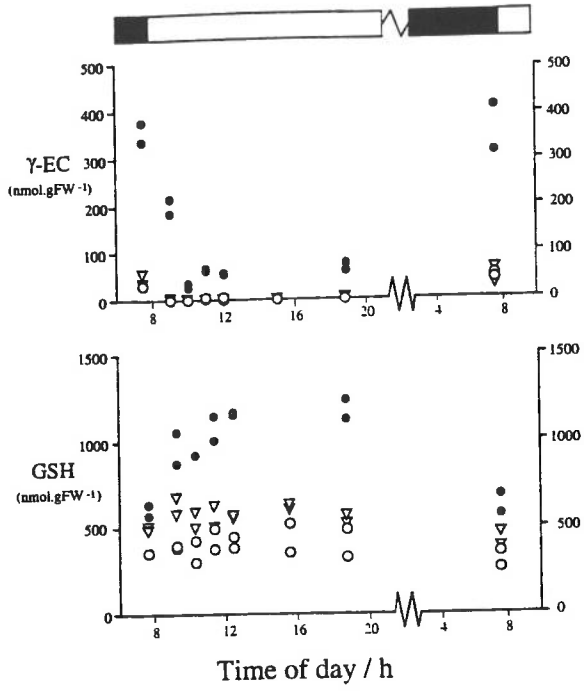


*Fig. 1.* Light-dark changes in γ-glutamylcysteine and glutathione in transformed and untransformed poplars. Closed circles, transformant gsh282d, overexpressing γ-ECS; open circles, untransformed; triangles, transformant 70gor, overexpressing glutathione reductase. Poplars were grown in a growth chamber under a light-dark regime of 16h:8h (22°C/16°C). Light intensity was 300 μmol $m^{-2}$ $s^{-1}$ at the leaf surface. Other experimental details as for Table 1.

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# REDUCED SENSITIVITY OF TRANSGENIC PLANTS EXPRESSING THE CYSTEINE SYNTHASE GENE TO SULPHUR DIOXIDE AND PARAQUAT

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## Abstract

Enhanced resistance of transgenic tobacco plants, expressing the wheat *cys1* gene, to either $SO_2$ fumigation or paraquat treatment, both of which result in the production of reactive oxygen species indicates a general increase in oxidative stress resistance of these plants. In addition to markedly increased cysteine, glutathione and sulphydryl levels, the plants also express high superoxide dismutase enzyme activities and transcript levels, which together may be responsible for the observed resistance character of these plants.

Transgenic tobacco plants (*Nicotiana tabacum*, cv Xanthi nc), expressing the wheat cysteine synthase gene (*cys1*), were previously shown to exhibit up to five-fold cysteine synthase activities and increased levels of resistance to $H_2S$ gas in comparison with control plants[1].

To evaluate the response of these plants to other sulphurous gases, 7-week-old plants (control, n=21; transgenic, n=25) were fumigated with 2 ppm $SO_2$ for 6 hr at 25°C, at a light intensity of 400 $\mu E\ m^{-2}\ s^{-1}$. After fumigation, plants were allowed to recover until the next day when injury to the leaves was visually assessed. Almost all the control plants showed extensive foliar damage, whereas the transgenic plants were almost unaffected by the treatment. More quantitative analyses, based on measurements of chlorophyll fluorescence, by which the quantum yields of electron transport by the reaction centre of photosystem II can be determined, and which serve as a measure of the photosynthetic activity and hence, in the current experiments, as an indicator of the sensitivity of the plants to $SO_2$ fumigation, confirmed the relative insensitivity of transgenic plants to fumigation. With respect to unfumigated plants, the control plants showed reductions in quantum yields of 21% and 77% after 1 and 2 ppm $SO_2$, respectively, whereas the transgenic plants showed respective reductions of only 11% and 37%.

As high levels of $SO_2$ are known to induce oxidative stress through the production of reactive oxygen species (ROS), the responses of the plants to paraquat (methyl viologen; MV), a chemical that generates ROS independently of the plant sulphur cycle, were tested. Leaf discs from transgenic and control plants were subjected to vacuum infiltration with different concentrations of MV for 18 hr in the light. Visualization of the discs from repeated experiments demonstrated that whilst the control leaf discs showed partial chlorosis at 0.2 mM MV and complete chlorosis at 0.5 mM, similar levels of chlorosis were

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only observed at 0.5 mM and 2.0 mM MV, respectively, in the transgenic leaf discs. In addition, electrolyte leakage experiments, performed essentially as described[2], in which leaf discs were infiltrated with 20 μM MV, washed in water, and placed in 10 ml of deionized water in the light and the conductivity of the solution measured at intervals, confirmed the significantly lower levels of MV-induced membrane damage in the transgenic plants.

In order to identify factors that may be involved in the resistance phenotype, the cysteine, SH and glutathione levels of the transgenic and control plants were determined as described[3]. Although contents of these tobacco plants were within previously reported ranges, the transgenic plants showed increases of about 65% in cysteine content, 24% in SH content and 13% in glutathione content over the control plant levels.

To test the possibility that other components of the oxidative stress response pathway were affected in the transgenic plants, Northern blot analysis was performed with tissues from control and transgenic plants exposed to different sulphur conditions, and probed with various SOD, catalase and peroxidase genes. Interestingly, the only consistent difference between the plants was a marked increase in cytoplasmic Cu/Zn SOD transcript levels in the transgenic plants under all conditions tested. This finding is consistent with the observation that the promoter of this cytoplasmic Cu/Zn SOD gene could be induced by cysteine or glutathione[4]. Furthermore, analysis of SOD activities in these plants also showed that the *cys1* transgenic plants had up to two-fold higher relative SOD activities than control plants.

Although the results clearly demonstrate an increase in resistance of the transgenic plants to oxidative stress, and point to some possible factors that may be involved in this process, the exact mechanisms which mediate these responses are still unclear. While it is possible that the increased cysteine content of the transgenic plants may well act as a reductant that is able to effectively scavenge free radicals, or that the increased glutathione content may more directly affect several enzymes involved in the pathway, or alternatively that the increased SOD activities may detoxify the radicals directly, the possibility of a more inclusive mechanism, in which all these factors effect a resistant phenotype in the transgenic plants, can not at present be excluded.

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# THE GLUTATHIONE STATUS DURING RECOVERY OF DESICCATED LICHENS: IS DESICCATION TOLERANCE CORRELATED WITH A HIGH REDUCING CAPACITY FOR GLUTATHIONE DISULPHIDE?

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## Abstract

Desiccating lichens for two days and two months, respectively, caused oxidation of reduced glutathione (GSH) in three lichen species with different desiccation tolerance[1]. Upon rehydration of two days desiccated lichens, oxidised glutathione (GSSG) was reduced rapidly in all three species. Wetting of two months desiccated thalli resulted in rapid reduction of GSSG in the most desiccation tolerant lichen, *Pseudevernia furfuracea* (L.) Zopf, and in *Lobaria pulmonaria* (intermediate) whilst its reduction showed a pronounced delay in *Peltigera polydactyla* (Necker) Hoffm., which is the most desiccation sensitive of these three species. The lichens might have obtained the NADPH required for the action of glutathione reductase (GR) (EC 1.6.4.2) from the oxidative pentose phosphate shunt. The activity of the key enzyme of this pathway, glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) was decreased dramatically by dehydrating *Peltigera polydactyla* and *Lobaria pulmonaria* for two months, while its activity was not affected in *Pseudevernia furfuracea*. Upon rehydration, the G6PDH-activity significantly increased in *Pseudevernia furfuracea* and in *Lobaria pulmonaria*, but not in *Peltigera polydactyla*. From these results we conclude that the reduction of GSSG in two months desiccated *Peltigera polydactyla* might be limited by the availability of NADPH.

Recently, the oxidation of reduced glutathione (γ-glutamyl-cysteinyl-glycine, GSH) to glutathione disulphide (GSSG) was reported during desiccation of three lichen species differing in their tolerance to desiccation[1]. *Pseudevernia furfuracea* was the most desiccation-tolerant species, *Peltigera polydactyla*, a lichen with cyanobacterial photobionts, the most desiccation-sensitive of these three species. *Lobaria pulmonaria*, like *Pseudevernia furfuracea*, a lichen containing green algae, had an intermediate position. Desiccation of all three lichen species led to oxidation of GSH from 10% up to 39% GSSG, expressed as a percentage of total glutathione (i.e. the sum of reduced and oxidised glutathione), after two days and up to 93% after two months of desiccation[1]. The oxidation of GSH was interpreted as an adaptation to drought following a thiol-disulphide cycle that was postulated recently[2]. This hypothesis depends upon the idea that protein thiol groups are protected from irreversible autooxidation by the reaction of GSSG with protein thiol groups (PSH) thus forming protein bound glutathione (PSSG) and GSH during desiccation. Upon extreme desiccation, GSH is oxidised further, leading to high final concentrations of PSSG and GSSG. In this state, PSH and GSH are protected from desiccation induced oxidative injury leading to irreversible formation of intramolecular cross links in proteins or uncontrolled oxidation of SH groups, e.g. to sulphonic acids. Lichens meet the demands of this hypothesis by containing enormous concentrations of GSSG when desiccated[1].

During recovery of resurrection plants, however, GSSG and PSSG must be reduced rapidly to yield the amounts of GSH and PSH that are required for the normal metabolic processes. Furthermore, GSSG probably inhibits protein synthesis[3,4,5]. Therefore, a rapid reduction to GSH is demanded for syntheses and growth. In this study, we investi-

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gated changes in the redox couple of glutathione during the rehydration of *Pseudevernia furfuracea* (L.) Zopf, *Lobaria pulmonaria* (L.) Hoffm. and *Peltigera polydactyla* (Necker) Hoffm. following a desiccation period of two days and two months, respectively. We determined changes in the activities of two enzymes needed for reduction of GSSG. These are glutathione reductase (GR) (EC 1.6.4.2) and glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49). The latter is the key enzyme of the oxidative pentose phosphate shunt that provides the NADPH required for the action of GR. We suppose this pathway is of special importance for resurrection plants that are not able to provide NADPH from photosynthesis in the first stages of recovery following desiccation.

Lichens were collected and methods used as previously described[1]. After desiccating lichens for two days or two months, they were rehydrated at 15°C in distilled water in the dark for 5, 15 and 60 min. The enzyme activities are expressed in nkat $g^{-1}$ DW. One katal is defined as the amount of GR/G6PDH that catalyses the oxidation / reduction of 1 mol NADPH + $H^+$ / $NADP^+$ $s^{-1}$.

During rehydration of two days desiccated lichens, GSSG was rapidly reduced in all three species. The activity of G6PDH significantly increased within 5 min of rehydration in *Pseudevernia furfuracea* and *Lobaria pulmonaria*, whilst in *Peltigera polydactyla* it tended to decrease. However, in this case the final enzyme activities (21 ± 4 nkat $g^{-1}$ DW for GR and 4 ± 0.5 nkat $g^{-1}$ DW for G6PDH) after 60 min seemed to be enough to maintain 93% of the total glutathione in the reduced state (Fig. 1). A rapid reduction of GSSG to GSH during the recovery of desiccated resurrection plants has been found by several authors[see 2]. It seems to be a feature of drought tolerant plants that they oxidise GSH during dehydration and reduce the GSSG so formed upon rehydration. While the oxidation of GSH may be interpreted as an adaptation mechanism that serves to protect GSH and PSH from desiccation induced oxidative injury[2], the reduction of the accumulated glutathione disulphide is necessary upon recovery to prevent GSSG mediated inhibition of protein synthesis. However, lichens desiccated for two months contained up to 93% of their glutathione in the oxidised form. This is, to the best of our knowledge, the highest amount of GSSG, expressed as a percentage of total glutathione, that has ever been described for a living organism. Wetting of two months desiccated thalli resulted in rapid reduction of GSSG in *Pseudevernia furfuracea* and *Lobaria pulmonaria*, whilst the reduction of GSSG in *Peltigera polydactyla* showed a pronounced delay compared to the other lichens (Fig. 1). We measured rather high GR activities during the rehydration of all three species, especially in *Peltigera polydactyla*, where the reduction of GSSG was far less efficient than in the two other lichens. We therefore conclude that a high activity of extractable GR is not a significant indicator of a high reducing capacity towards GSSG. Consequently, the reduction of GSSG could be limited by the availability of NADPH. This was proved by measuring the activity of G6PDH during desiccation[1] and rehydration of the lichens. G6PDH activity was decreased dramatically by desiccating thalli of *Lobaria pulmonaria* and *Peltigera polydactyla* for two months, while its activity was not affected in *Pseudevernia furfuracea*[1]. During rehydration, the G6PDH-activity significantly increased in *Lobaria pulmonaria* from 1 ± 0.4 nkat $g^{-1}$ DW to 9 ± 5 nkat $g^{-1}$ DW within 5 min of rehydration. This was sufficient to reduce GSSG from 93% of total glutathione, to 18%. In *Peltigera polydactyla*, however, G6PDH activity increased from 1 ± 0.2 nkat $g^{-1}$ DW to a maximum of only 2 ± 1 nkat $g^{-1}$ DW (not significantly different) after 15 min and decreased again to 1 ± 0.5 nkat $g^{-1}$ DW after 60 min of rehydration. In spite of the high GR activities during rehydration (about 20 nkat $g^{-1}$ DW), GSSG (71% of total glutathione after 2 months of desiccation) was only reduced to 30% after 60 min of wetting in water. Moreover, we observed a loss of total glutathione in *Peltigera polydactyla* that was not measured in the two green algae containing lichens, *Pseudevernia*

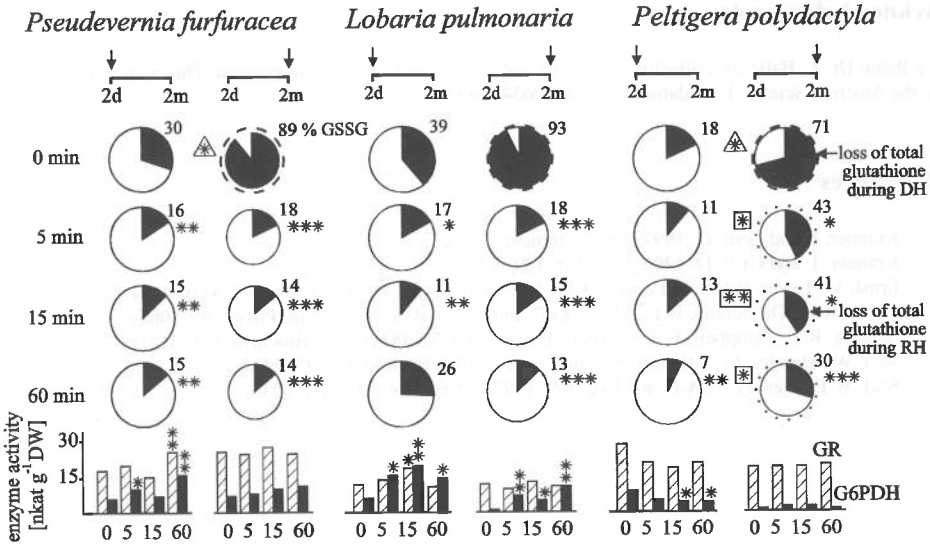


*Fig. 1.* Reduction of GSSG (black segments) and enzyme activities of GR (hatched bars) and G6PDH (black bars) during rehydration of three lichen species following a desiccation period of 2 days (2d) and 2 months (2m). The total glutathione contents (expressed by circles) after 2 days of desiccation of *Pseudevernia furfuracea* (2372 $g^{-1}$ DW), *Lobaria pulmonaria* (2473 nmol $g^{-1}$ DW) and *Peltigera polydactyla* (1982 nmol $g^{-1}$ DW) are calculated as 100%, GSSG is given as a percentage of total glutathione. The rehydration times listed on the left relate to changes in the GSH/GSSG ratio, those relating to the changes in enzyme activities are given at the bottom. The loss of total glutathione between 2d and 2m of desiccation is expressed by smaller circles, and the original glutathione content of 2d desiccated lichens is indicated by a broken line. A loss of total glutathione during rehydration was only observed in *Peltigera polydactyla* after 2months of desiccation. Here, the original glutathione content of 2m desiccated thalli is indicated by a dotted line. Data were tested for significance with Kruskall-Wallis rank variance analysis and, thereafter, Dunn's test. Significant differences (* $P<0.05$, ** $P<0.005$, *** $P<0.001$) between desiccated and rehydrated thalli concerning GSSG, GR and G6PDH are indicated by stars. Stars included in triangles refer to changes in the total glutathione content between 2d and 2m of desiccation. Significant changes in the total glutathione content during the rehydration of 2m desiccated thalli of *Peltigera polydactyla* are indicated by stars included in rectangulars. DH, dehydration; RH, rehydration.

*furfuracea* and *Lobaria pulmonaria*. This fits the results of other authors who correlated the maintenance of antioxidants, among them GSH, with a high capacity for scavenging desiccation induced free radicals. The formation and accumulation of stable free radicals was reported e.g. in a desiccation tolerant moss (*Tortula ruraliformis*) and in a desiccation sensitive moss (*Dicranella palustris*)[6]. In these mosses, maintenance of GSH was observed only in *Tortula ruraliformis*, while GSH was depleted in *Dicranella palustris*. The loss of glutathione we observed during the rehydration of two months desiccated *Peltigera polydactyla* probably indicates a high demand for GSH. Moreover, we conclude that these three lichens show a degree of desiccation tolerance that could be correlated with their capability to reduce GSSG upon rehydration following desiccation. After rehydration of two months desiccated *Peltigera polydactyla* the reduction of GSSG seems to be limited by the availability of NADPH that is not provided sufficiently by the oxidative pentose shunt.

## Acknowledgements

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# DESICCATION OF LICHENS: CHANGES IN THE GLUTATHIONE STATUS

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## Abstract

Glutathione was oxidised in three lichen species differing in their desiccation tolerance up to 39% of the total glutathione content after two days and up to 93% after two months of desiccation. Simultaneously, all three species showed a marked loss of glutathione after being desiccated for two months. The activity of glutathione reductase (GR) (EC 1.6.4.2) was not seriously affected after two months of desiccation in all three lichens. However, the activity of glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) was dramatically decreased in two species that are less tolerant to desiccation (*Lobaria pulmonaria* (L.) Hoffm. and *Peltigera polydactyla* (Necker) Hoffm.) than the most desiccation-tolerant species, *Pseudevernia furfuracea* (L.) Zopf. This species did not show an effect of desiccation on this enzyme.

Glutathione (γ-glutamyl-cysteinyl-glycine) fulfils significant biological functions in catalysis, synthesis and transport[1,2], most of them being ascribed to reduced glutathione (GSH) that is recycled by the enzyme glutathione reductase (GR) (EC 1.6.4.2) after its oxidation to glutathione disulphide (GSSG). In most tissues glutathione is maintained in the reduced state, and the accumulation of GSSG is therefore commonly correlated with functional disorder. However, a central role for GSSG was suggested recently for overcoming resting stages of plant development including dormancy of seeds and desiccation of resurrection plants. Here, the accumulation of GSSG and protein-bound glutathione was introduced as an important mechanism that serves in protecting thiol groups from desiccation induced oxidative injury in desiccation tolerant plants[3]. As GR is an NADPH-dependent enzyme, the creation of NADPH by the oxidative pentose phosphate pathway attracts interest for investigating the redox status of glutathione under non-photosynthetic conditions. This is the case during dormancy and the first stages of germination of seeds, and desiccation and recovery of resurrection plants.

We investigated changes in the redox status of glutathione and the activities of GR and glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) – the key enzyme of the oxidative pentose shunt – during desiccation of three lichens differing in their desiccation tolerance: *Pseudevernia furfuracea* (L.) Zopf is the most desiccation-tolerant species; *Peltigera polydactyla* (Necker) Hoffm., a lichen with cyanobacterial photobionts, seems to be the most desiccation-sensitive of these three species. *Lobaria pulmonaria* (L.) Hoffm., like *Pseudevernia furfuracea* a lichen containing green algae, has an intermediate position.

*Pseudevernia furfuracea* (L.) Zopf growing on *Picea abies* 1550 m above sea level was collected at the Salzstiegl, Austria. *Lobaria pulmonaria* (L.) Hoffm. and *Peltigera polydactyla* (Necker) Hoffm. were collected at the Sneznik, Slovenia, in 1100 m above sea level., *Lobaria pulmonaria* growing on *Acer sp.* or *Fraxinus sp.*, and *Peltigera polydactyla* growing on the soil. These lichens were put into a desiccator over silica gel for 2 days and 2 months, respectively. GSH and GSSG[4], GR and G6PDH activities[5] were assayed as previously described.

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During desiccation, the GSH present in lichens in their natural habitat was oxidised in all species investigated. After 2 days of desiccation, GSSG was enhanced from the control content (10-23%) to 18-39% of total glutathione in all three species. Desiccating lichens for 2 months resulted in loss of glutathione and, simultaneously, a further increase in its oxidised form in all three lichens. Two months desiccated thalli contained between 71-93% of the total glutathione in the oxidised form (Fig. 1).

The loss of GSH after two months of desiccation might be explained by GSH consuming processes such as scavenging of desiccation-induced free radicals. The accumulation of free radicals in dehydrated tissues was reported be several authors[see 6]. However, the accumulation of GSSG fits the hypothesis that glutathione is involved in protecting protein thiol groups from desiccation-induced autooxidation. The changes in enzyme activities (Fig 1) of GR and G6PDH also meet the demands of this hypothesis:

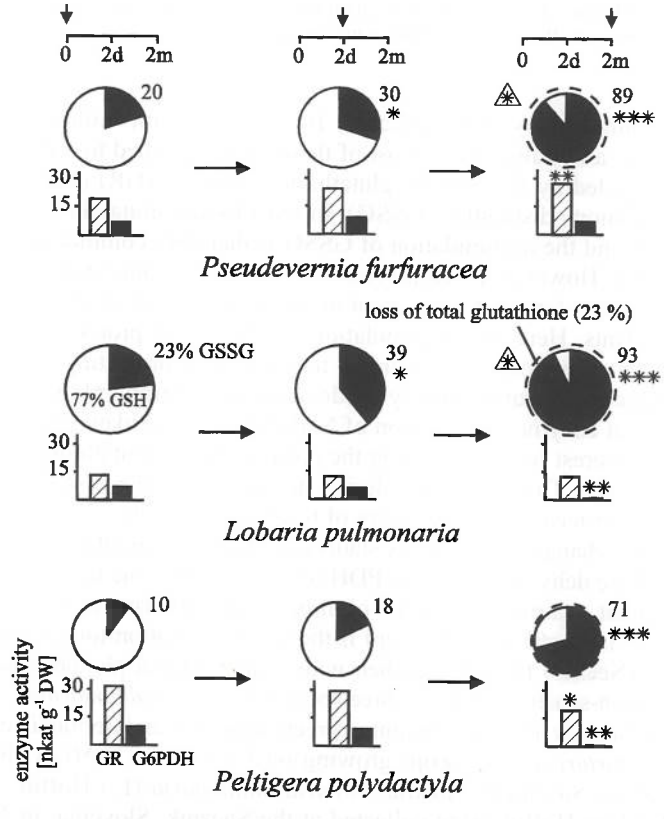


*Fig. 1.* Changes of GSH (white segments) and GSSG (black segments) and enzyme activities of GR (hatched bars) and G6PDH (black bars) during desiccation of three lichen species. The duration of desiccation is given on top. 0, 2 d, 2 m: zero (controls), 2 days and 2 months period of desiccation. The total glutathione content of *Lobaria pulmonaria* (2792 nmol $g^{-1}$ DW) is taken as 100%. The lower total glutathione contents of *Pseudevernia furfuracea* (2313 nmol $g^{-1}$ DW) and *Peltigera polydactyla* (1735 nmol $g^{-1}$ DW) are expressed by smaller circles. Data were tested for significance with Kruskall-Wallis rank variance analysis and, thereafter, Dunn's test. Significant differences (* $P<0.05$, ** $P<0.005$, *** $P<0.001$) between the controls and the desiccated thalli concerning GSSG, GR and G6PDH are indicated by stars. Stars included in triangles refer to changes between the controls and the desiccated thalli in the total glutathione content.

*Pseudevernia furfuracea* seemed to activate the enzymes required for a rapid reduction of GSSG by increasing the activity of GR during two months of desiccation while the activity of G6PDH was not affected. In *Peltigera polydactyla* the activity of GR tended to decrease within two months, however, the activity of G6PDH declined dramatically. *Lobaria pulmonaria* revealed its intermediate position by an unchanged GR but a drastically decreased G6PDH activity when desiccated for two months. Consequently, an adaptation to drought can be correlated with the mobilisation of these enzymes, GR and G6PDH, needed for the reduction of the GSSG formed under desiccating conditions.

## Acknowledgements

We thank Dr. F. Batic for collecting *Peltigera polydactyla* and *Lobaria pulmonaria*. This work was supported by the Austrian Science Foundation, grant P 10634-BIO.

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# CONSEQUENCES OF CHRONIC OZONE EXPOSURE IN TRANSGENIC POPLARS OVEREXPRESSING ENZYMES OF THE GLUTATHIONE METABOLISM

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## Abstract

Transgenic poplar trees overexpressing different enzymes of the glutathione metabolism, namely glutathione synthetase, glutathione reductase and γ-glutamyl-cysteine synthetase (γ-EC synthetase) were subjected to chronic ozone exposure. After four weeks of fumigation, all lines clearly showed visible damage, compared to controls fumigated with ambient air. However, none of the transgenic lines overexpressing the different enzymes of glutathione metabolism showed enhanced resistance towards ozone. Even the line overexpressing γ-EC synthetase, which had about 2-fold enhanced glutathione contents, did not show a lower level of damage upon ozone exposure. There was no change in glutathione contents due to ozone fumigation. Apparently, enhanced glutathione content or elevated glutathione reducing capacity does not protect poplar trees from damage derived from chronic ozone exposure. Glutathione does not seem to play a crucial role in detoxifying reactive oxygen species under these conditions.

Ozone is one of the most widespread air pollutants in the industrialised world, causing visible damage as well as metabolic changes in plants[1]. It reacts instantaneously with components of the cell wall and the plasma membranes, thereby forming reactive oxygen species such as superoxide radicals, hydroxyl radicals and hydrogen peroxide. Numerous studies have shown that cellular glutathione levels change upon exposure to oxidative stress[2,3] and various attempts have been made to improve resistance to oxidative stress by overexpressing either superoxide dismutase, glutathione reductase or glutathione synthetase[4]. In the present investigation the question was addressed as to whether transgenic poplar trees overexpressing different enzymes of glutathione metabolism show different reactions or even enhanced resistance to chronic ozone stress.

The present study was performed with poplar plants (*Populus tremula x P.alba)*, overexpressing glutathione reductase either in the cytosol or in the chloroplast, or glutathione synthetase in the cytosol or γ-EC synthetase in the cytosol. For this purpose, the plants were transformed by co-cultivation with *Agrobacterium tumefaciens* containing the coding sequences of the respective bacterial enzymes, controlled by the 35S promoter of the CaMV. For expression in the chloroplast, the pea rbcS coding sequence of a transit peptide was fused to the vector[5,6]. The plants were micro-propagated and cultivated in the greenhouse for about 60 days. Subsequently, the young poplar trees were exposed for four weeks to ozone concentrations as measured in July 1990 near Basel (Switzerland). The average daily maximum gas mixing ratio of ozone was 94.1 nl $l^{-1}$. The plants were illuminated for 14 hours per day with ca. 300 μE photosynthetically active radiation

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(PAR), at 70% relative humidity and at a temperature of 25°C (day) and 20°C (night). Visible damage of the first 25 leaves of each plant was assessed by an independent viewer as percentage of affected leaf area. Glutathione contents were determined using reverse phase HPLC as described previously [7].

Despite the relatively low ozone concentration, leaves of all polar plants clearly showed symptoms of damage after four weeks of fumigation (Table 1). This damage appeared as blurred or sharply defined dark spots, mainly on leaves of an age between two and four weeks, which were exposed to ozone during their entire lifetime.

None of the transgenic poplar lines overexpressing the different enzymes of glutathione metabolism showed a lower level of damage after ozone exposure. Even those plants overexpressing γ-EC synthetase with glutathione contents about 2-fold higher than all other lines (Table 2) did not show increased resistance towards ozone. Despite clearly visible signs of damage, the rates of photosynthesis and plant growth were not affected during the four weeks exposure. Since there also was no difference in stomatal conductance, the leaves did not prevent ozone exposure by reducing gas exchange (data not shown).

The levels of glutathione in the different lines were about the same as previously reported[5,6]. The glutathione content in the line overexpressing γ-EC synthetase was twice as high as in all other lines[8]. There was no significant change in the glutathione content due to ozone exposure in any of the transgenic poplar lines studied (Table 2). The ratio of oxidized glutathione was about the same in all lines (2 – 4 % of total glutathione) and did not change upon ozone fumigation (data not shown). From these findings we conclude that increased glutathione contents or enhanced capacity for glutathione reduction does not protect poplar plants against damage from chronic ozone stress. Obviously, the glutathione content is not a limiting factor in the plants' defence system against oxidative stress derived from ozone exposure.

*Table 1.* Visible damage in fumigated and control plants. The data shown are means and standard deviations of four independent assessments. Significant differences at $p<0.05$ are indicated by different letters

| *Enzyme overexpressed* | *Leaf damage (% leaf area)* | |
|---|---|---|
| | *$O_3$ – fumigated* | *ambient air* |
| Untransformed | $4.6 \pm 2.9^{b}$ | $0.1 \pm 0.1^{a}$ |
| γ-EC – Synthetase (cytosolic) | $9.0 \pm 2.4^{c}$ | $2.5 \pm 0.4^{ab}$ |
| GSH – Synthetase (cytosolic) | $9.2 \pm 2.6^{c}$ | $0.7 \pm 1.1^{a}$ |
| GSH – Reductase (cytosolic) | $8.4 \pm 3.0^{c}$ | $0.1 \pm 0.1^{a}$ |
| GSH – Reductase (chloroplastic) | $8.1 \pm 1.6^{c}$ | $2.3 \pm 1.4^{ab}$ |

*Table 2.* Total glutathione contents (GSH + GSSG) of leaves from fumigated and unfumigated plants. After four weeks of fumigation, leaves which were the 3rd from the top at the beginning of fumigation were analysed. The data shown are means and standard deviations of four independent analysis. Significant differences at $p<0.05$ are indicated by different letters

| *Enzyme overexpressed* | *Glutathione content (nmol g fresh weight$^{-1}$).* | |
|---|---|---|
| | *$O_3$ – fumigated* | *ambient air* |
| Untransformed | $256 \pm 56^{ab}$ | $327 \pm 20^{a}$ |
| γ-EC – Synthetase (cytosolic) | $583 \pm 64^{c}$ | $626 \pm 206^{c}$ |
| GSH – Synthetase (cytosolic) | $312 \pm 19^{ab}$ | $318 \pm 19^{ab}$ |
| GSH – Reductase (cytosolic) | $256 \pm 17^{ab}$ | $290 \pm 51^{a}$ |
| GSH – Reductase (chloroplastic) | $412 \pm 64^{b}$ | $388 \pm 44^{b}$ |

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# SEASONAL CHANGES IN GLUTATHIONE S-TRANSFERASE ACTIVITY OF *PINUS SYLVESTRIS* AND *P. NIGRA* NEEDLES

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## Abstract

Glutathione S-Transferase (GST) activity was measured for two consecutive years in 1-year old needles of *Pinus sylvestris* and *P. nigra* from a 30-year old forest stand exposed continuously to severe drought and high temperature in summer. The stand is located near the river Rhein in Hartheim, Germany. Both species showed highest GST activity in 1-year-old needles in summer on a fresh weight and protein basis. Since glutathione in the conifers showed relatively low levels in summer, increasing GST activities may be required for maintaining GST mediated reactions at this time of the year. GST is thought to play a role in the metabolism and detoxification of lipid peroxidation products that may be of particular significance under severe drought and high temperature in summer. Nevertheless, no activity of GST in *Pinus* was found using cumene hydroperoxide or 13 (s)-Hydroperoxy-(9Z,11E)-octadecadienoic acid as a substrate. There was also no correlation between GST activity and lipid peroxidation measured as malondialdehyde content. From these results it appears doubtful that GST is involved in the detoxification of lipid peroxidation products in pine trees.

Glutathione S-transferases (GSTs, E.C. 2.5.1.18) are a family of enzymes which catalyze the conjugation of glutathione with electrophilic, hydrophobic compounds. However, the natural functions of GSTs are still not known. GST is thought to play a role in the metabolism and detoxification of lipid peroxidation products[4]. A GST preparation from pea, for example, catalyzes the detoxification of linoleic acid hydroperoxide to an alcohol[6].

*Pinus sylvestris* is a typical stress-enduring species. It can grow under several stressful conditions including drought, and high and low temperatures. Little is known about GST of trees exposed to stress conditions. In this study, we analyzed GST activity in the needles of *P. sylvestris* and *P. nigra* in a forest stand exposed continuously to severe drought and high temperatures in summer.

Needles of *P. sylvestris* and *P. nigra* from a 30-year-old forest located near the river Rhein in Hartheim, Germany were collected during different times of the season in 1994 and 1995. GST was extracted with 0.15M TRIS-HCl buffer containing 3% soluble PVP, 5mM EDTA and 3mM GSH, pH 7.8. Crude extracts were purified on Sephadex G-25 columns and then used for the determination of GST activity and protein contents. GST activitiy was measured with glutathione and CDNB as substrates as described by Habig et al.[3]. Protein was quantified as described by Bradford[1] using BSA as a standard. Glutathione extraction procedures and HPLC analysis were performed according to Schupp and Rennenberg[5], lipid peroxide measurement according to Esterbauer[2].

Maximum GST activity both in current year and 1-year-old needles of *P. sylvestris* was observed in early August, 1994 and in middle July, 1995. The activity in current year needles was 9 times higher in summer than in winter on a protein basis and 3.8 times on a fresh weight basis (Fig. 1. A, C). In 1-year-old needles, this ratio was 6.9 times on a protein basis and 2.5 times on a fresh weight basis. Apparently, the differences in GST activ-

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ity between summer and winter can partially be attributed to general changes in protein contents rather than specific changes in GST activity.

In *P. nigra*, a similar seasonal pattern of GST activity was observed for current year needles in 1994 and 1-year old needles in 1995 (Fig. 1. B). On a fresh weight basis, the GST activity in current year needles showed no common pattern among 5 individual trees in 1995. On a protein basis, the activity in current year needles showed two peaks, one in mid July and the other in September (Fig. 1. D). Since the protein level both, in current year and 1-year-old needles was lower during the growing season than in winter (Fig. 1. E, F), the GST activity was higher in summer than in winter on a protein basis (Fig. 1.C, D). The protein level was higher in 1-year-old needles than in current year needles throughout the year. This also reflected on GST activity which on a protein basis always showed higher activities in current year needles than in 1-year-old needles (Fig. 1. C, D). Thus, a major part of the seasonal change in GST activity can be explained by a general change in protein content.

The GST activity of the samples collected on July 14 was measured with cumene hydroperoxide or 13 (s)-Hydroperoxy-(9Z,11E)-octadecadienoic acid as a substrate. In *Pinus* no GST activity was found with these substrates. Lipid peroxidation was measured by malondialdehyde with TBA. There was also no correlation between GST activity and the lipid peroxide levels during the season (data not shown). From these findings it can be assumed that GST is not involved in the detoxification of lipid peroxification products in pine trees. Total glutathione and oxidized glutathione were relative low in summer and lowest from the end of August to early September (Fig. 2.). Therefore, increasing GST activities may be required in summer for maintaining GST mediated reactions at this time of the year.

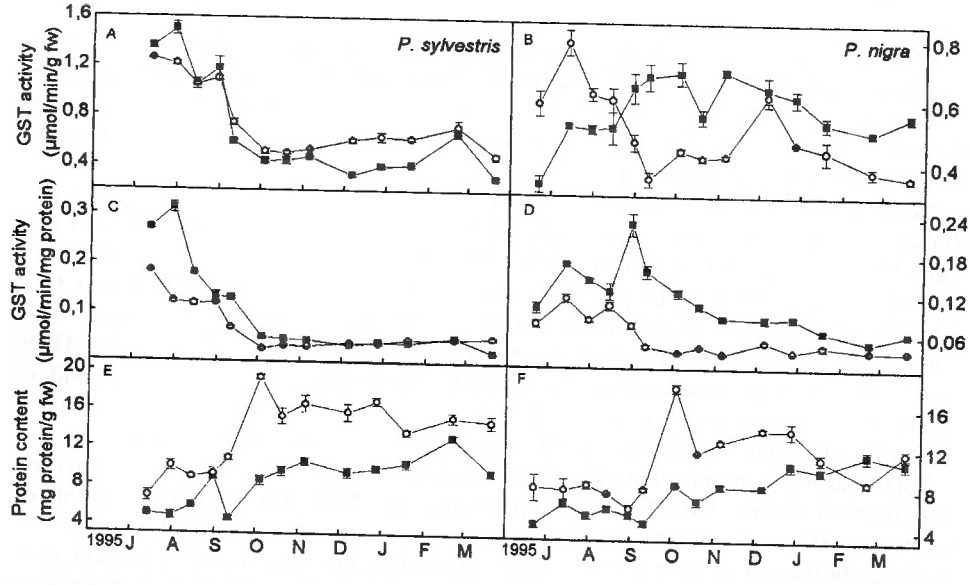


*Fig. 1.* Seasonal changes of GST activities and protein contents in *Pinus sylvestris* and *P. nigra* needles. (—■—) current year needles, (—○—) 1-year-old needles.

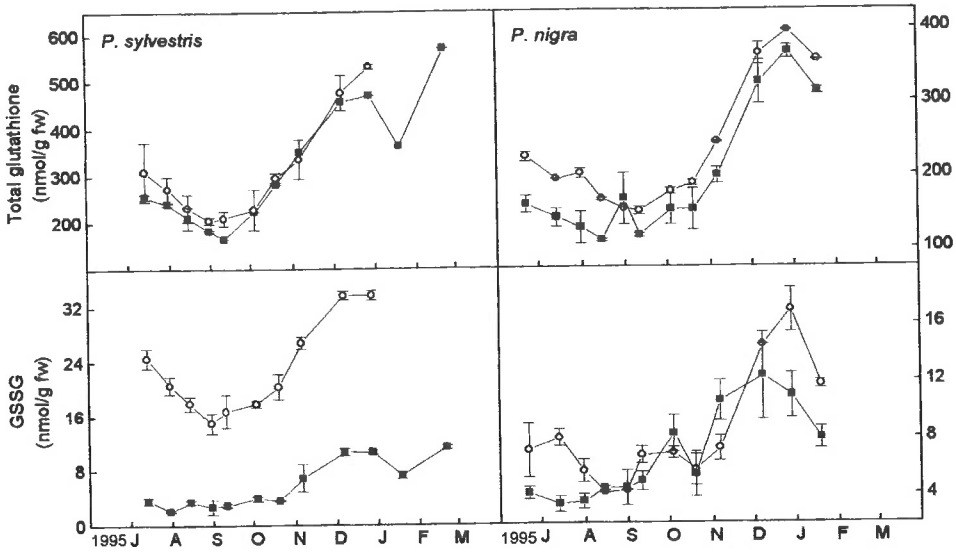


*Fig. 2.* Seasonal changes of total glutathione and oxidized glutathione in *Pinus sylvestris* and *P. nigra* needles. (-■-) current year needles, (-○-) 1-year-old needles.

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# TOLERANCE TO PHOTOOXIDATIVE STRESS OF TRANSGENIC TOBACCO PLANTS WITH ALTERED ACTIVITY OF GLUTATHIONE REDUCTASE

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## Abstract

Transgenic tobacco plants which have enhanced activities of glutathione reductase (GR) (EC 1.6.4.2) and/or superoxide dismutase (SOD) (EC 1.15.1.1) were resistant to paraquat. The leaves of these transgenic plants had higher contents of both reduced glutathione (GSH) and ascorbate than those of the control plants during paraquat treatment (10μM) in the light. These transgenic plants showed no more resistance to an air pollutant ozone than the control in terms of the extent of visible foliar damage, although GSH and ascorbate contents in the transgenic leaves were higher than those of the controls after 2h-exposure to 0.3ppm ozone.

Active oxygen species such as superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide ($H_2O_2$) are generated as by-products in many biological reactions. They are toxic to living organisms and, unless removed rapidly by a scavenging system of cells, destroy various cellular components and/or inactivate metabolism. The generation of active oxygen species is promoted under environmental conditions such as drought[1], low temperature[2], and exposure to air pollutants[3] or some herbicides[4] in the light, which are thought to cause photooxidative damage to plants. There are enzymes in plants responsible for scavenging active oxygen species and these enzymes are thought to be involved in the photooxidative stress tolerance of plants[5,6]. Glutathione reductase (GR) (EC 1.6.4.2) is one of these enzymes and is postulated to supply the reduced form of glutathione (GSH) to the ascorbate-glutathione cycle[7].

The role of GR in plant tolerance to photooxidative stress was analyzed using four types of transgenic tobacco plants: 1) those containing the GR of *Escherichia coli* origin in the cytosol[8]; 2) those containing the *E. coli* GR in the chloroplasts[9]; 3) those having antisense spinach cDNA for GR[10]; 4) those with the GR of *E. coli* and rice superoxide dismutase (SOD), that is also one of the enzymes for scavenging active oxygen, in the cytosol[11]. Transgenic tobacco with high cytosolic [1)] or chloroplastic [2)] GR activity showed enhanced tolerance to photooxidative stress caused by a herbicide paraquat or an air pollutant sulphur dioxide[8,9]. Transgenic tobacco with reduced activity of GR [3)] exhibited enhanced sensitivity to paraquat[10]. In addition, transgenic tobacco with simultaneously enhanced activities of GR and SOD [4)] showed extremely high tolerance to paraquat[11]. These results indicate that plant tolerance against photooxidative stress is dependent on the activities of these antioxidant enzymes and that co-operative work of these enzymes is important.

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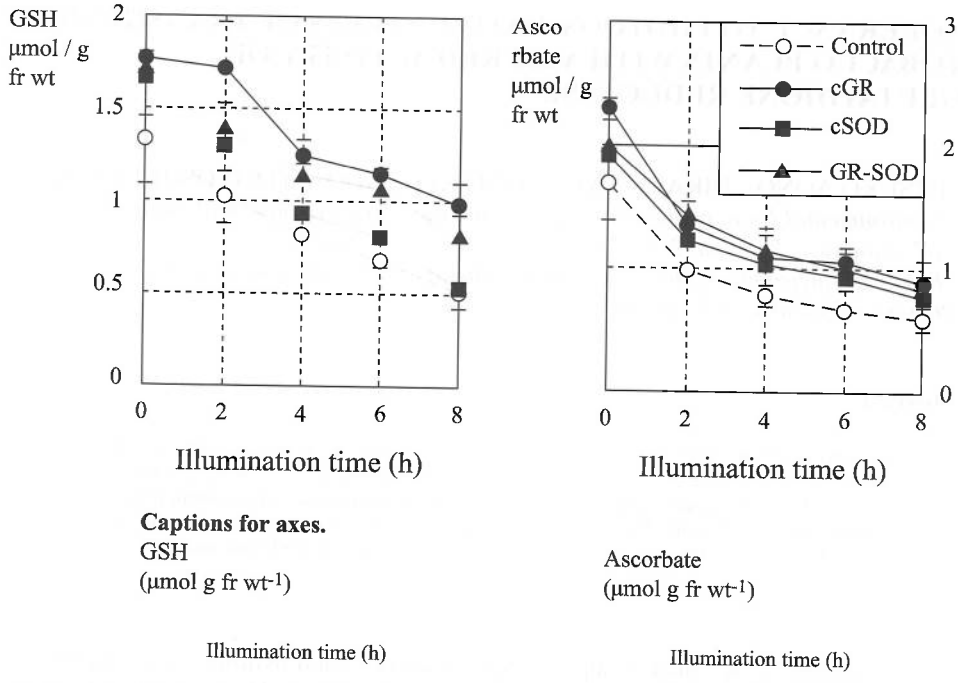


*Fig. 1.* Change of the contents of GSH and ascorbate in leaves of the transgenic and the control tobacco plants during paraquat treatment. Tobacco plants were grown on a regime of 14 h light (130μE $m^{-2}$ $s^{-1}$) and 10 h dark at 25°C for 6 weeks. Twenty leaf discs were excised from a young-expanded leaf. Five individuals were used per one type of tobacco for an experiment. The leaf discs were supplied with 10μM paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride; Sigma) with 0.1% Tween 20 by vacuum infiltration. They were washed and transferred to deionized water in a Petri dish and were exposed to light (130 μE $m^{-2}$ $s^{-1}$) at 25°C for 8 h. Twenty discs (0.4 g) were collected every 2 h and homogenised in 2 volumes (0.8 ml) of 5% meta-phosphate followed by a centrifugation at 6000 x g for 10 min. Contents of GSH and ascorbate in the supernatant were measured by colorimetric assay using Bioxytech® GSH-400 (OXIS International S. A., France) and a reflecting photometer (RQflex and Reflectquant Ascorbate test, Merck, Germany). Each point was the average of results from three independent experiments. Vertical bars stand for SE. Control – non-transgenic SR1; cGR – transgenic tobacco with enhanced cytosolic activity of GR[8]; cSOD – transgenic tobacco with enhanced SOD activity[12]; GR-SOD – transgenic tobacco with enhanced activities of both GR and SOD[11].

The transgenic tobacco with high GR and /or SOD activity had higher foliar contents of both GSH and ascorbate than the control during paraquat treatment in the light (Fig. 1). Hence, the higher content of GSH is thought to enhance regeneration of ascorbate for raising the capacity of scavenging active oxygen in the transgenic cell. However, these transgenic plants exhibited no more resistance to an air pollutant ozone than the control in terms of the extent of visible foliar damage, although GSH and ascorbate contents in the transgenic leaves were higher than those of the controls after the exposure to ozone (Table 1). These results suggest that the mechanism of injury caused by ozone may be different from that caused by paraquat.

*Table 1.* Effect of ozone on the contents of GSH and ascorbate in leaves of the transgenic and the control tobacco plants. Six-week-old tobacco plants were exposed to 0.3 ppm ozone for 2 h at 25°C in the light (400 μE $m^{-2}$ $s^{-1}$) in a growth cabinet (230 × 190 × 170 $cm^3$). The same series of tobacco plants were placed in another growth cabinet without ozone as a control (0 ppm ozone). Ozone exposure was performed as described in ref 8. Ten leaf discs were excised from a young-expanded leaf and GSH and ascorbate contents were measured (see legend for *Figure 1*). Control, cGR, cSOD and GR-SOD; see legend for *Figure 1*. Values are average of those from five individuals and ± SE.

| | | GSH (μmol/g fr $wt^{-1}$) | %[a] | Ascorbate (μmol/g fr $wt^{-1}$) | %[a] |
|---|---|---|---|---|---|
| Control | 0 ppm ozone | 1.10 ± 0.04 | | 2.69 ± 0.00 | |
| | 0.3 ppm | 0.94 ± 0.03 | 85.5 | 0.33 ± 0.01 | 12.3 |
| cGR | 0 ppm | 1.03 ± 0.00 | | 2.70 ± 0.07 | |
| | 0.3 ppm | 1.17 ± 0.01 | 113.6 | 0.61 ± 0.02 | 22.6 |
| cSOD | 0 ppm | 0.96 ± 0.02 | | 1.91 ± 0.04 | |
| | 0.3 ppm | 1.04 ± 0.00 | 108.3 | 0.53 ± 0.07 | 27.7 |
| GR-SOD | 0 ppm | 0.95 ± 0.02 | | 1.91 ± 0.06 | |
| | 0.3 ppm | 1.03 ± 0.03 | 108.4 | 0.84 ± 0.05 | 44.0 |

[a] The ratio (%) of the value for 0.3 ppm ozone exposure to the value for 0 ppm ozone

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# INCREASED CAPACITY FOR GLUTATHIONE BIOSYNTHESIS IN THE CHLOROPLAST PARADOXICALLY PROMOTES OXIDATIVE STRESS IN TRANSGENIC TOBACCO

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## Abstract

We have transformed tobacco with genes encoding the two enzymes of glutathione biosynthesis. We report here that these plants indeed make more glutathione and γ-glutamylcysteine, but paradoxically the plants appear to suffer oxidative stress.

The most relevant functions of reduced glutathione (GSH) in the context of oxidative stress are the ascorbate-glutathione cycle of plants[1] and the redox cycle involving GR and glutathione peroxidase activity associated with true peroxidases and some classes of glutathione-S-transferases[2]. GSH participates in a variety of other reactions such as the glyoxylase system which reduces α-ketoaldehydes to α-hydroxycarboxylic acids, in the reduction of ribonucleotides to deoxyribonucleotides and in thiol:disulphide exchange reactions[5]. Thus GSH has been implicated in the regulation of enzyme activities, in DNA synthesis, in maintaining the viability of chloroplasts and mitochondria and in the regulation of gene expression possibly via thiol-sensitive transcription factors[3-6]. Given these beneficial roles for GSH, we have set out to elevate the levels of GSH in plants and this paper reports first results of attempts to determine the effects of this on their ability to withstand oxidative stress.

We have transformed tobacco with chimaeric genes encoding γ-glutamylcysteine (γ-EC) synthetase (*gshI*) and glutathione (GSH) synthetase (*gshII*) from *E. coli* and the products of these genes were targeted to the chloroplast. Plants overexpressing *gshII* are phenotypically normal and do not have increased GSH levels. However, plants expressing *gshI* have up to 6-fold higher GSH and up to 11-fold higher γ-EC levels (Table 1). We have studied five independent transgenic lines and in all cases the plants display leaf necrosis and chlorosis to varying degrees, the symptoms of which correlate with the expression of the *gshI* transgene. Elevated $H_2O_2$ concentrations (2-fold), depressed $CO_2$ fixation rates, and pathological changes in chloroplasts from prenecrotic leaves indicate that the plants are suffering from internally generated oxidative stress. However, there were no changes in the foliar amounts of lipid peroxidation products, oxidatively damaged proteins or ascorbate levels. The same enzyme activities elevated in the cytosol do not produce these symptoms, indicating that the effects are localised in the chloroplast.

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*Table 1.* Foliar amounts of GSH and γ-EC in F2 progeny of crosses between two independent *gshI* tobacco lines (B8 and B24) and one *gshII* line (D1). The values are the means (± standard error) of sample sizes shown in the "n" column. Plants were grown in a controlled environment room at 25°C/22°C, 16 h photoperiod, PFD 250 mmol $m^{-2}$ $s^{-1}$ , and 70% relative humidity and were sampled at 6 weeks post-germination. Leaves were either at node 5 or 6.

| | GSH | γ-EC | n |
|---|---|---|---|
| | nmol/g fresh wt | | |
| *gshI*-B8 | 915 ± 165 | 117 ± 27 | 12 |
| *gshII*-D1 | 584 ± 176 | 14 ± 10 | 3 |
| *gshI*-B8 x *gshII*-D1 | 3361 ± 581 | 128 ± 46 | 6 |
| transgene recessive | 650 ± 102 | 11 ± 7 | 9 |
| *gshI*-B24 | 781 ± 194 | 29 ± 8 | 6 |
| *gshII*-D1 | 663 ± 156 | 10 ± 3 | 12 |
| *gshI*-B24 x *gshII*-D1 | 1550 + 293 | 33 ± 5 | 16 |
| transgene recessive | 557 ± 118 | 5 ± 2 | 12 |

These symptoms can be partially alleviated by producing hybrids in which both enzymes of GSH biosynthesis are overexpressed. In the hybrids the levels of GSH are elevated up to 5-fold and there is a slight reduction in γ-EC levels (Table 1).There was an associated decline on the foliar levels of $H_2O_2$ in parallel with the diminution of symptoms. Interestingly, none of the genes encoding enzymes which scavenge active oxygen species (AOS) or catalyse redox reactions with ascorbate or glutathione in the chloroplast or the cytosol are induced in these stressed plants. These data suggest that γ-EC, in some way, interferes with the sensing of internally generated AOS causing an accumulation of AOS as by-products of photosynthetic activity, the effects of which, in the *gshI/gshII* hybrids, are offset by the elevated levels of glutathione.

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# INVOLVEMENT OF CYTOCHROME P450 IN OXIME PRODUCTION IN GLUCOSINOLATE BIOSYNTHESIS

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## Abstract

In vitro enzyme systems for the conversion of amino acid to oxime in the biosynthesis of glucosinolates have been established by the combined use of an improved isolation medium and jasmonic-acid induced seedlings of *Sinapis alba L.* and *Tropaeolum majus L.* The oxime production has been demonstrated to involve cytochrome P450s as shown by photoreversible CO-inhibition in *S.alba* and cytochrome P450 inhibitors in *T.majus*.

Glucosinolates are amino acid-derived secondary plant products found throughout the order Capparales, which includes the family Brassicaceae with the agriculturally important crop oilseed rape. The presence of glucosinolates in this plant is of economical importance due to the potentially harmful effects of their breakdown products. Glucosinolates are grouped into aliphatic, aromatic and indolyl glucosinolates depending on whether they are derived from methionine, phenylalanine and tyrosine, or tryptophan[1]. The parent amino acid often undergoes a series of chain elongations prior to entering the biosynthetic pathway, and the glucosinolate product is often subject to secondary modifications like hydroxylations, methylations, oxidations, etc. *In vivo* biosynthetic studies using seedlings or excised tissues have demonstrated that *N*-hydroxyamino acids, nitro compounds, oximes, thiohydroximates, and desulfoglucosinolates are precursors of glucosinolates[1].

We have chosen *Sinapis alba* L. (Brassicaceae) and *Tropaeolum majus* L. (Tropaeolaceae) as model plants for studying the biosynthesis of glucosinolates. Both plants have only one major glucosinolate derived from the amino acid tyrosine and phenylalanine, respectively. *Brassica napus* L. is less suitable as model plant because it contains several glucosinolates derived primarily from chain-elongated protein amino acids not commercially available. *In vitro* biosynthetic studies have been hampered by low biosynthetic activities and by the release of inhibitory degradation products from endogenous glucosinolates during tissue homogenization. The latter was monitored by preparing microsomes from seedlings from *Sorghum bicolor* L. in the presence of leaves from *T. majus* L.[2]. When prepared separately, the sorghum microsomes were actively synthesizing *p*-hydroxymandelonitrile in the biosynthesis of the cyanogenic glucoside dhurrin. The biosynthetic activity of the sorghum microsomes were inactivated by the *T. majus* leaves. The inhibitory component was shown to be the benzylisothiocyanate produced by myrosinases during homogenization[2].

Recently, we have obtained a 6-8 fold increase in the level of *de novo* biosynthesis of glucosinolates in seedlings of *S. alba* and *T. majus* by treatment of the seedlings with 50 μM jasmonic acid in 50% EtOH (Fig.1)[3,4]. The *in vivo* biosynthetic activities were measured as incorporation of $^{14}$C-tyrosine and $^{14}$C-phenylalanine, respectively, into the corre

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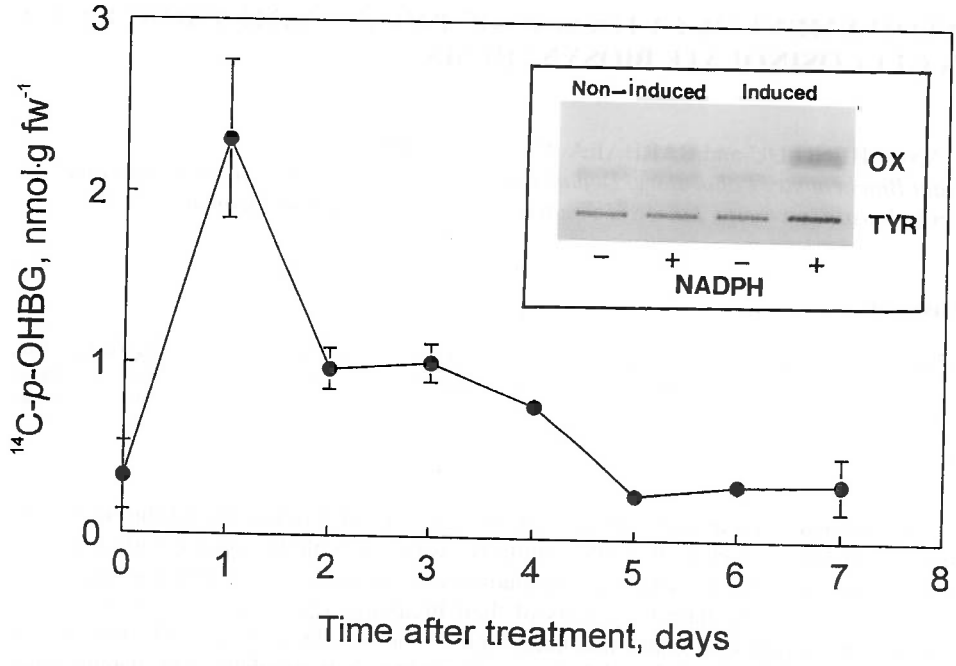


*Fig. 1.* Induction of *de novo* biosynthesis of *p*-OHBG in 3-day old seedlings of *S. alba* upon treatment with jasmonic acid. The biosynthetic activity was measured as incorporation of radioactivity into *p*-OHBG upon administration of [U-$^{14}$C]tyrosine to the seedlings. Insert: Production of *p*-hydroxyphenylacetaldoxime from tyrosine by the *S. alba* microsomal enzyme system. Standard reaction mixtures were incubated with 2.2 nmol of $^{14}$C-tyrosine for 30 min at 35°C. *p*-Hydroxyphenylacetaldoxime accumulated in the reaction mixtures as evidenced by comparison with authentic standards in TLC. Abbreviation: fw, fresh weight; OX, *p*-hydroxyphenylacetaldoxime; TYR, tyrosine.

sponding glucosinolates upon administration of the tracers to excised seedlings. In addition, we have isolated biosynthetically active microsomal enzyme systems catalyzing the conversion of amino acids to oximes from the jasmonic acid-treated seedlings (Fig.1, insert). The inhibitory effects of the degradation products was partly prevented by adding 100 mM ascorbic acid and 5 mg/ml bovine serum albumin to the isolation buffer. Ascorbic acid is known to inhibit myrosinase at high concentrations and the bovine serum albumin was found to protect the biosynthetic enzymes. Under these conditions approximately 50% of the sorghum biosynthetic activity was recovered after homogenisation of sorghum seedlings in the presence of leaves of *T. majus*.

Knowledge about the enzymes catalyzing the biosynthesis of glucosinolates is only slowly emerging. The last two steps in the pathway are catalyzed by two soluble enzymes, *i.e.* the UDPG:thiohydroximate glucosyltransferase, which glucosylates the thiohydroximate, and the PAPS:desulfoglucosinolate transferase, which converts desulfoglucosinolate into the glucosinolate[5]. These two enzymes have been purified and shown to be unspecific with respect to the nature of the side chain. The sulphur-donating enzyme has not been characterized, but feeding experiments suggest that cysteine is the sulphur donor[6]. The nature of the enzymes catalyzing the conversion of amino acids to oximes is the subject of many discussions. Recently, we have shown photo-reversible carbon monoxide inhibition of the conversion of tyrosine to *p*-hydroxyphenylacetaldoxime by *S. alba* microsomes (Table 1)[3]. This demonstrates that a cytochrome P450 is

*Table 1.* Photoreversible carbon monoxide inhibition of the production of *p*-hydroxyphenylacetaldoxime by *S. alba* microsomes

| Experimental conditions | | Oxime production (%) |
|---|---|---|
| $O_2$ | - light | 100 |
| $O_2$ | + light | 86 |
| $CO + O_2$ | - light | 18 |
| $CO + O_2$ | + light | 62 |

The microsomal reaction mixtures containing 200 nmol of $^{14}C$-labelled tyrosine were incubated under the experimental conditions indicated as described under "Material and Methods". After incubation 30 min at 35°C, the reaction mixtures were extracted with ethyl acetate and the produced oxime was separated from other components by TLC and quantified by liquid scintillation counting. 100% activity represents 344 *p*mol oxime mg protein$^{-1}$ h$^{-1}$. Abbreviation: $O_2$ – normal atmosphere ($O_2$:$N_2$ ::20:80, v/v); $CO + O_2$ – atmosphere composed of CO:$O_2$:$N_2$ (10:10:80, v/v/v).

involved. Similarly, we have shown that the conversion of phenylalanine to phenylacetaldoxime in *T. majus* microsomes is inhibited by cytochrome P450 inhibitors, indicating that the phenylacetaldoxime production is dependent on cytochrome P450[4]. This shows that in two different families within the order Capparales the conversion of amino acids to oximes is catalyzed by cytochrome P450s.

Cyanogenic glucosides are a related group of secondary plant products, which likewise have amino acids as precursors and oximes as intermediates[7]. In the biosynthesis of dhurrin, the tyrosine-derived cyanogenic glucoside in *S. bicolor*, a single, multifunctional cytochrome P450 has been shown to catalyze the conversion of tyrosine to *p*-hydroxyphenylacetaldoxime[8]. Our results on cytochrome P450 involvement in the oxime production by microsomes from *T. majus* and *S. alba* provide experimental documentation that homologous enzyme systems catalyze the conversion of amino acids into the corresponding oximes in the biosynthesis of cyanogenic glucosides and glucosinolates.

Wallsgrove and co-workers have demonstrated NADPH-dependent $CO_2$ release and oxime production from homophenylalanine by microsomes isolated from young leaves of *Brassica napus* L.[9,10]. The ability to measure NADPH-dependent release of $CO_2$ was extended to several aliphatic and aromatic amino acid precursors of glucosinolates in oilseed rape and other *Brassica* species[10,11]. The decarboxylation reactions were shown not to be sensitive to the presence of carbon monoxide or the cytochrome P450 inhibitor ABT, indicating that cytochrome P450 was not involved in these reactions[11]. Wallsgrove and coworkers suggested that the conversion of aromatic and aliphatic amino acids to oximes in *Brassica* species was catalyzed by flavin monooxygenases[10]. Ludwig-Müller and co-workers have indications that the oximes in the biosynthesis of indolylglucosinolates in *Brassica* species are synthesized by a plasmamembrane-bound peroxidase-type of enzyme[12,13]. If all these sets of observations are correct, this would imply that the enzyme system catalyzing the conversion of amino acids to oximes within the Brassicaceae family has evolved independently three times.

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# MOLYBDENUM COFACTOR BIOSYNTHESIS: IDENTIFICATION OF *A. THALIANA* cDNAS HOMOLOGOUS TO THE *E.COLI* SULPHOTRANSFERASE MoeB

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## Abstract

Based on the deduced amino acid sequence of the *E.coli* MoeB-sulphotransferase, a protein which is involved in the molybdopterin-biosynthesis, we have identified an *Arabidopsis* EST with significant amino acid homology. Alignments to all MoeB-homologues available in data-bases and subsequent phylogenetic analysis gave evidence of four eukaryotic homologues of *E.coli* MoeB forming an evolutionary group distinct from a number of other prokaryotic proteins with still significant but lower amino acid homology to MoeB. All these members are nevertheless thought to catalyse the transfer of reduced sulphur in different pathways. They all share a common ancestor and have evolved divergently subsequent to several gene duplication events. In addition to several highly conserved amino acid residues all proteins show a common sequence motif containing two identical cysteine-clusters which are probably involved in complexing metals.

The Molybdenum cofactor (Moco) is an essential component common to all molybdoenzymes with the exception of nitrogenase. Nitrate reductase is the key plant molybdoenzyme followed by xanthine dehydrogenase and aldehyde oxidase whereas sulphite oxidase and retinal oxidase are important representatives of molybdoenzymes in animals. Due to the pleiotropic loss of all molybdoenzymes, a defective Moco has lethal or sublethal consequences for the organism. Moco mutants (in eukaryotes *cnx* for "cofactor of nitrate reductase and xanthine dehydrogenase") are described in bacteria, fungi, algae, mosses, higher plants, insects and humans.

This presentation focuses on the isolation and characterization of eukaryotic homologues of the *E.coli* MoeB sulphotransferase in plants, a protein which is thought to be involved in the insertion of sulphur into the Moco-precursor via the molybdopterin-synthase complex[4,5]. We have sequenced a full-length *Arabidopsis* cDNA-clone encoding a protein that is closely related to a human MoeB-homologue and shows significant amino acid homology to *E.coli* MoeB. In association with two fungal proteins these four members form a distinct eukaryotic group. Thus they are very probably eukaryotic equivalents of *E.coli* MoeB and therefore thought to be directly involved in Moco-biosynthesis. The eubacterial proteins HesA[1,6] and MccB share a significantly lower homology to both the *E.coli* MoeB and its eukaryotic homologues, and therefore they are considered as outgroups. The remaining ThiF protein which is involved in thiamine biosynthesis[7] is located between the MoeB cluster on the one hand and the outgroups on the other hand with significantly higher homology to MoeB (see fig.1 and table1). A second *Arabidopsis* cDNA-clone has been identified due to amino acid homology to MoeB, but homology is restricted to a rather short but highly conserved N-terminal sequence motif ressem-

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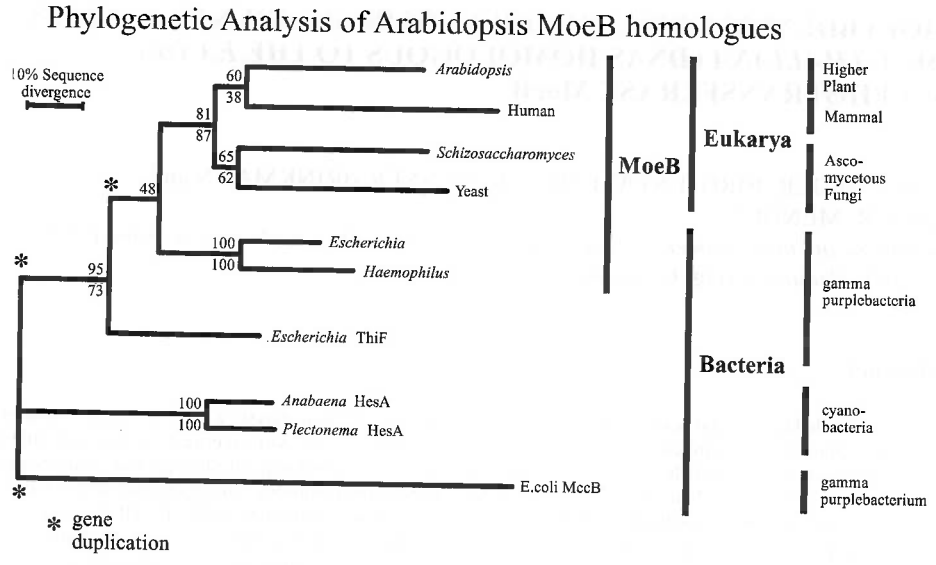


*Fig. 1.* Phylogenetic analysis of MoeB homologous proteins based on amino acid sequences. The tree has been constructed using the neighbour-joining method of the program package ClustalV based on the corrected percent amino acid identity[3]. The numbers given above the branches are bootstrap values obtained in 100 bootstrap replica, the numbers given below the branches are bootstrap values obtained in 100 replica using parsimony of the program package PAUP (protein analysis using parsimony)[2]. The four eukaryotic proteins form a distinct group separated from the prokaryotic MoeB-representatives of *E.coli* and *Haemophilus influenzae* with ThiF sharing significant homology to both the eukaryotic and prokaryotic proteins, whereas HesA and MccB show low homology to MoeB, thus forming outgroups.

*Table 1.* Presentation of the deduced amino acid identity[2,3] between *Arabidopsis thaliana* MoeB and its homologues available in the data bases. The values are calculated according to the ClustalV program[2,3]. Individual percentage identity values are shown above the shaded bars while the numbers of amino acid residues compared are listed below. *Arabidopsis thaliana* MoeB is closely related to all other eukaryotic representatives with a high score of identity (41%) between *A. thaliana* MoeB and the human homologous protein while the prokaryotic MoeB-proteins (Eco and Hae.) share 63% of amino acid identity. The HesA-proteins and *E.coli* MccB are regarded as outgroups whereas *E.coli* ThiF shows significant homology to both the eukaryotic and the prokaryotic MoeB.

Abbreviations: *Arabidopsis thaliana* (Ath.), Human (Hum.), *Schizosaccharomyces pombe* (Spom.), *Saccharomyces cerevisiae* (Yst), *Escherichia coli* (Eco), *Haemophilus influenzae* (Hae.), *Anabaena sp.* PCC7120 (Ana.), *Plectonema boryanum* (Plec.).

| | Ath. MoeB | Hum. MoeB | Spom. MoeB | Yst. MoeB | Eco. MccB | Eco. MoeB | Hae. MoeB | Eco. ThiF | Ana. HesA | Plec. HesA |
|---|---|---|---|---|---|---|---|---|---|---|
| Ath.MoeB | – | 41 | 40 | 39 | 21 | 40 | 39 | 41 | 32 | 33 |
| Hum.MoeB | 325 | – | 40 | 36 | 20 | 40 | 30 | 38 | 28 | 29 |
| Spom.MoeB | 388 | 287 | – | 42 | 21 | 37 | 39 | 41 | 31 | 30 |
| Yst.MoeB | 424 | 305 | 397 | – | 25 | 36 | 38 | 36 | 27 | 27 |
| Eco.MccB | 315 | 184 | 265 | 292 | – | 24 | 25 | 27 | 26 | 26 |
| Eco.MoeB | 247 | 168 | 248 | 247 | 212 | – | 63 | 46 | 33 | 36 |
| Hae.MoeB | 241 | 162 | 442 | 241 | 206 | 243 | – | 40 | 31 | 30 |
| Eco.ThiF | 249 | 173 | 250 | 250 | 210 | 245 | 239 | – | 35 | 37 |
| Ana.HesA | 262 | 200 | 246 | 258 | 202 | 227 | 221 | 231 | – | 72 |
| Plec.HesA | 273 | 198 | 249 | 262 | 216 | 233 | 227 | 234 | 258 | – |

bling a different enzyme-species, the so-called ubiquitine activating enzymes. The observed homology to MoeB might be due to their function of transferring reduced sulphur to the ubiquitine conjugating enzymes.

The amino acid alignment of all MoeB-proteins and their homologues shows a striking difference in length between the eubacterial and the eukaryotic proteins. The latter reveal approximately 150 additional amino acids at the carboxy-terminus with respect to *E.coli* MoeB, suggesting an additional function.

A large number of amino acid residues are highly conserved amongst all organisms, being either structural elements or involved in the catalytic function. Almost all proteins show two cysteine-clusters separated by two amino acids each, a sequence motif which is thought to ensure metal-binding. Although complexed metals are more likely to be a component of all sulphotransferases in general[8] it remains to be examined whether these cysteines might enable additional binding of pathway-specific metals like molybdenum

Due to the participation of several enzymes in Moco-biosynthesis that are at least in part homologous to other sulphur-transferring enzymes of different metabolic pathways and due to the fact that the element sulphur itself is an important component of Moco, we hope to find an interaction between sulphur- and nitrogen-metabolism at the level of Moco-biosynthesis.

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# DIMETHYLSULPHONIOPROPIONATE IN *SPARTINA*-GRASSES

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## Abstract

Although DMSP is often referred to as being an osmolyte, no proof for that function exists in *Spartina*. Of the factors investigated so far (salinity, sulphate, sulphide, nitrogen), only nitrogen has been shown to strongly affect DMSP concentrations in the plants. A pilot experiment in which DMSP, proline and glycinebetaine in plants grown at different levels of salinity and nitrogen supply were analysed by HPLC suggests a negative correlation between DMSP and glycinebetaine in *Spartina*.

Dimethylsulphoniopropionate (DMSP) has been detected in high concentrations in higher plants only in species of the genera *Spartina*, *Saccharum* and *Wollastonia*[2,4,5,6]. In *Spartina* it occurs in *S. alterniflora, S. townsendii*, *S. maritima* and *S. foliosa*, but has not been detected *in S. pectinatus*, *S. cynosuroides* and *S. patens*[4]. In the *Spartina*-species that produce DMSP in significant quantities, the highest concentrations are always found in green leaf tissue. The compound has been suggested to act as an osmolyte, but so far no relation has been shown between exposure of the plants to salinity and concentrations of DMSP in the plant tissues[4,6]. Exposure to sulphur as sulphate or sulphide does not affect concentrations of DMSP either. However, in the absence of salt in the growth medium, DMSP concentrations in the plants as well as the amount of total sulphur allocated to DMSP decrease with increasing nitrogen supply to the plants[4].

Recent research approaches in our laboratory, aimed at elucidating the location and functions of DMSP in *Spartina*, involve X-ray microprobe analysis by electron microscope and consecutive analysis of DMSP, glycinebetaine and proline by HPLC. Preliminary data obtained by X-ray microprobe analysis suggest that high sulphur densities, most of which would be expected to be in the form of DMSP[4], are associated with chloroplasts in leaf tissue[3].

In a pilot experiment, plants were grown on solutions containing 0, 400 or 800 mM NaCl and 0 or 2 mM $NH_4NO_3$ (Table 1). Proline levels were increased at 800 mM NaCl compared to the lower salinities, regardless of the level of $NH_4NO_3$. Glycinebetaine and DMSP concentrations in the leaves did not vary consistently with treatments, but concentrations of DMSP appeared negatively correlated with concentrations of glycinebetaine (Figure 1, product-moment correlation coefficient of log-transformed values = -0.481, borderline significant at $0.05<P<0.1$).

The response of proline to salinity corresponds with the findings of Cavalieri[1] who showed that proline accumulated in *Spartina alterniflora* when the plants were exposed to salinities higher than 600 mM (approximately the salinity of seawater). The trend of a negative relationship between concentrations of DMSP and glycinebetaine may indicate a shift in compounds involved in osmotic regulation from N-based to S-based compounds.

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*Table 1.* Means ± standard deviations of concentrations of DMSP, glycinebetaine and proline in green leaf tissue of *Spartina townsendii* plants grown in treatment solutions (sand culture) containing concentrations of NaCl and $NH_4NO_3$ as indicated

| Treatment | | | DMSP | Glycinebetaine | Proline |
|---|---|---|---|---|---|
| NaCl/mM | $NH_4NO_3$/mM | n | μmol $g^{-1}$ fw | μmol $g^{-1}$ fw | μmol $g^{-1}$ fw |
| 0 | 0 | 3 | 48 ± 33 | 45 ± 25 | 5.4 ± 1.4 |
| 0 | 2 | 4 | 12 ± 4 | 50 ± 10 | 4.2 ± 4.3 |
| 400 | 0 | 2 | 8.3 ± 0.4 | 66 ± 30 | 5.3 ± 1.1 |
| 400 | 2 | 4 | 27 ± 24 | 29 ± 8 | 6.8 ± 3.3 |
| 800 | 0 | 1 | 34 | 38 | 12 |
| 800 | 2 | 1 | 38 | 39 | 15 |

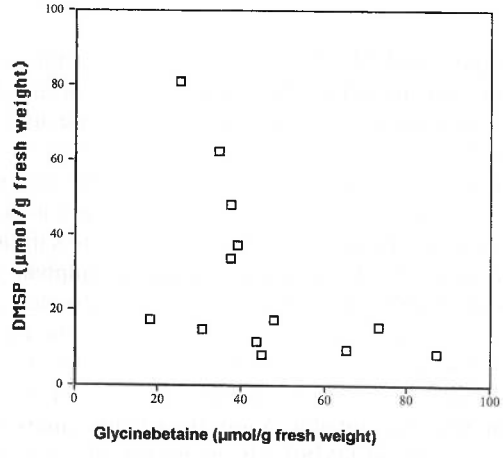


*Fig. 1.* Relationship between concentrations of DMSP and glycinebetaine (μmol $g^{-1}$ fresh weight) in *S. townsendii*. Pooled values for all treatments.

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# THE DEPOSITION AND EMISSION OF SULPHUR COMPOUNDS BY CROPS.

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## Abstract

The deposition to, and efflux from, wheat of the atmospheric sulphur gases carbonyl sulphide, hydrogen sulphide, dimethyl sulphide, carbon disulphide and methyl mercaptan, were investigated under controlled conditions. Atmospheric air was supplemented with extra carbonyl sulphide to twice the background concentration for a short time during the experimental period. The magnitude of the deposition and efflux was dependent upon the age and the nitrogen status of the crop. Carbonyl sulphide concentration in the atmosphere also influenced these processes. These data are discussed in the context of the global sulphur cycle.

The flux of sulphur gases to and from vegetation is one of the major uncertainties of the global sulphur cycle[1]. Carbonyl sulphide (COS) is the principle sulphur gas in the atmosphere with a concentration of 500 ppt and has been proposed as an important climatic gas because it is a source of stratospheric submicron aerosol particles[2]. The oceans are a major source of dimethyl sulphide ($[CH_3]_2S$)(DMS)[3] as are rice paddy fields[4]. Although significant emissions of DMS by trees have been reported their contributions to the global S-budget seem to be low[5]. The other gaseous sulphur species of interest are hydrogen sulphide ($H_2S$), methyl mercaptan ($CH_3SH$), and carbon disulphide ($CS_2$), all of which have been shown to exchange with vegetation[6,7].

Plants were grown either in a complete Hoagland's solution or in a low nitrogen Hoagland's solution, 15mM and 7mM of nitrogen respectively, in a controlled environment cabinet at 20°C and 50% RH. At 7a.m. on the day of exposure plants were transferred to a teflon lined chamber (0.5m × 0.5m × 0.5m) and the air flow through the chamber, 8 l $min^{-1}$, was started. The inlet contained 47 ppt of $CS_2$ and 340 ppt of COS, the air was humidified to 60% RH by passing through a water reservoir. At the same time the air stream was supplemented with COS from a cylinder to raise the chamber concentration to 1400 ppt.

A 1500 ml sample of the air entering and exiting the chamber was taken by cryotrapping at 100 ml $min^{-1}$ and used for gas chromatographic analysis as described by Hofmann *et al.*[7]. The gas sampling began at 9 a.m. and continued until 11 a.m. The additional COS was then switched off. After an hour for plant acclimation the air sampling began again at the ambient COS concentration. Two hours later sampling was stopped and the plants were removed from the chamber and harvested. Data from these analyses were used to calculate fluxes of the sulphur gases to or from the crop.

With increasing age the plants changed from net emission of sulphur to net deposition of sulphur (Table 1). The net sulphur deposition was greatest when the COS entering the

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*Table 1.* Flux of compounds from wheat during exposure to ambient and elevated levels of COS (negative values indicate deposition)

| Nitrogen level | Crop age (days) | COS level | Gaseous fluxes (ng $m^{-2}$ $min^{-1}$) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | $H_2S$ | COS | $CH_3SH$ | DMS | $CS_2$ | S balance |
| Normal | 6 | elevated | 0.37 | -0.77 | 0 | 0.14 | -0.04 | -0.01 |
| | | ambient | 0.14 | -0.17 | 0 | 0.05 | 0 | 0.07 |
| | 7 | elevated | 0.23 | -1.81 | 0 | 0.09 | 0.05 | -0.62 |
| | | ambient | 0.17 | -0.18 | 0 | 0.15 | 0.06 | 0.19 |
| | 19 | elevated | -0.23 | -14.99 | 0 | -0.16 | -0.91 | -8.8 |
| | | ambient | -0.31 | -2.77 | 0 | -0.18 | -0.88 | -2.56 |
| Low | 8 | elevated | 0.16 | -0.51 | 0 | 0.26 | 0.1 | 0.1 |
| | | ambient | 0.54 | 0.08 | 0 | 0.26 | 0.18 | 0.83 |
| | 16 | elevated | 0.1 | -4.71 | 0.01 | 0.28 | 0.03 | -2.16 |
| | | ambient | 0.15 | -0.65 | 0.12 | 0.22 | 0.03 | 0.02 |

chamber was above the ambient atmospheric concentration and on those occasions when there was a net efflux of sulphur the values were lower during elevated COS treatment. This was paralleled by the behaviour of COS deposition which increased with plant age and increasing plant nitrogen status.

The flux of $H_2S$ went from emission in the young plants, 6 and 7 days old, to deposition in the 19 day old plants. Plants grown in the low nitrogen solution also reduced their emissions of $H_2S$ with plant age, but unlike the plants grown in the complete solution there was no deposition of $H_2S$ to older plants. This pattern of behaviour was also observed for DMS. A similar pattern was also observed with $CS_2$, with the exception of 6 day old plants growing in the complete solution. A flux of $CH_3SH$ was only observed in the older plants grown in low nitrogen medium, when an efflux was recorded.

Linear regression analysis was used to investigate the relationship between the rates of emission of $H_2S$, DMS, $CS_2$ and $CH_3SH$, and the deposition of COS. The correlation was not significant ($p>0.05$).

Increasing deposition of COS to agricultural plants with increasing mixing ratio has also been observed by Kesselmeier and Merck[9], who reported a linear increase in the deposition of COS for maize and rape, for mixing ratios above the compensation point of 100 ppt. Hydrogen sulphide emission from young wheat plants has also been previously observed[10], but in these experiments DMS was emitted at all plant ages. What is notable in these experiments is that the emission of these gases was enhanced by the elevated COS level, although a significant relationship between COS deposition and emission of the other gases measured was not be found. Any correlation may have been disguised by the differences in age and nitrogen status of the plants.

With respect to global sulphur budgets the experiments support the hypothesis that vegetation is a major sink for COS[11]. However, when calculating the total sulphur deposition both the age and the nitrogen status of the vegetation need to be taken into account. Further experiments for the parameterisation of these factors are now required.

## Acknowledgement

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# UPTAKE OF ATMOSPHERIC $H_2S$ BY *SPINACIA OLERACEA* L. AND CONSEQUENCES FOR THIOL CONTENT AND COMPOSITION OF SHOOTS AND ROOTS

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## Abstract

Upon exposure to atmospheric $H_2S$ total water-soluble non-protein thiol content increased in the roots and the shoots of spinach plants. In the shoot this increase was partly due to elevated cysteine levels, whereas in the roots cysteine contents were not affected. After three hours of exposure approximately 30 % of the $H_2S$ taken up could be found in the thiol fraction.

Plant shoots form a sink for atmospheric $H_2S$ and the rate of uptake is determined by its direct metabolism[4,5]. Cysteine synthase appears to be directly involved in the active uptake of $H_2S$ by the plant[4,5]. $H_2S$ exposure results in a rapid accumulation of water-soluble non-protein thiols in the shoots[2,3,4,5]. In the present communication the relation between uptake of $H_2S$ and thiol accumulation is evaluated.

Spinach plants that had been grown for 9 days on a ¼-strength Hoagland solution[1] in a climate-controlled room (photoperiod 12 hours at a photon fluence rate of 350 μmol $m^{-2}$ $s^{-1}$ [400-700 nm], day and night temperatures of 23°C and 20°C, respectively) were exposed to atmospheric $H_2S$ for up to 48 h. Cysteine and total water-soluble non-protein thiol contents were determined as described by De Kok *et al.*[2], sulphate contents as described by Maas *et al.*[3]. $H_2S$-uptake rate was derived from the differences in concentrations between the inlet and outlet ports of a 2.5 l glass cuvette with stainless steel bottom. Similar to previous observations[4,5], the rate of uptake of $H_2S$ by spinach shoots showed saturation kinetics with respect to the atmospheric $H_2S$ concentration, which could be described by Michaelis-Menten kinetics. A 48 hour exposure of plants to 0.06 or 0.3 μl $l^{-1}$ $H_2S$ did not significantly affect the uptake kinetics of $H_2S$ by the shoots (Fig. 1). The apparent maximum uptake rate $J(H_2S)_{max}$ was 0.17 nmol $cm^{-2}$ $min^{-1}$ (0.5 μmol g $FW^{-1}$ $h^{-1}$) and the apparent $k[H_2S]$ (concentration at which ½ $J(H_2S)_{max}$ was reached) was 0.4 μl $l^{-1}$. After the first 3 hours of exposure approximately 30% of the $H_2S$ taken up by the shoots could be revealed in the thiol fraction (Table 1). Apparently the greater proportion was further metabolized and used as sulphur source for plant growth.

Exposure of spinach plants to 0.06 and 0.3 μl $l^{-1}$ $H_2S$ resulted in a rapid accumulation of total water-soluble non-protein thiol compounds in the shoot (Fig. 2A). Maximal accumulation of thiols was observed after 24 hours of exposure, irrespective of the atmospheric $H_2S$ concentration. Part of this accumulation was due to an enhanced cysteine content (Fig. 2C). After some delay there was also an increase of the thiol level in the roots upon $H_2S$ exposure (Fig. 2B), which was similar at 0.06 and 0.3 μl $l^{-1}$ $H_2S$ and lower than that observed in the shoots. The cysteine content in the roots was not affected by $H_2S$ expo-

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sure (Fig. 2D). This supports the idea that cysteine is not transported from the shoot to the roots and that glutathione is the main transport form of reduced sulphur in the plant[6]. The sulphate content in the shoot was not substantially affected by $H_2S$ exposure (Fig. 2E), whereas in the roots there was a slight increase in sulphate content after 24 and 48 hours of exposure (Fig. 2F). The latter may indicate that sulphate uptake by the roots (partially) continues after $H_2S$ exposure, but its transport to the shoot is reduced.

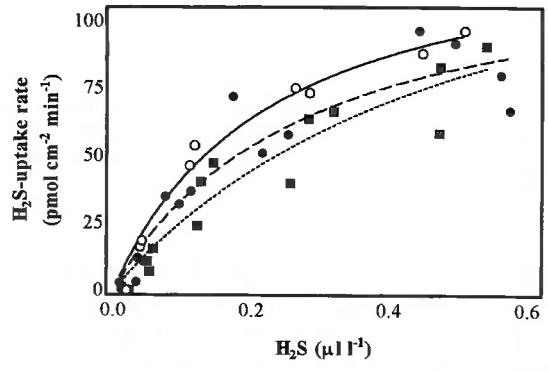


*Fig. 1.* $H_2S$ uptake by shoots of *Spinacia oleracea* L. at various atmospheric $H_2S$ concentrations. Plants were pre-exposed to 0 (○—○), 0.06 (●—●) and 0.3 µl $l^{-1}$ $H_2S$ (■—■) for 48 hours before the uptake measurements. $H_2S$ exposure did not affect the transpiration rate. The $H_2S$-uptake rate was determined in triplicate using three plants per measurement.

*Table 1.* Uptake of $H_2S$ by shoots versus thiol accumulation in Spinacia oleracea during the first 3 hours of exposure to $H_2S$

| | $H_2S$ supply | |
|---|---|---|
| | 0.06 µl $l^{-1}$ | 0.3 µl $l^{-1}$ |
| $H_2S$ taken up (µmol g fresh weight$^{-1}$ 3h$^{-1}$) | 0.261 | 1.103 |
| Thiol accumulation (µmol g fresh weight$^{-1}$ 3h$^{-1}$) | 0.078 | 0.317 |
| $H_2S$ in thiol fraction (%) | 30 | 29 |

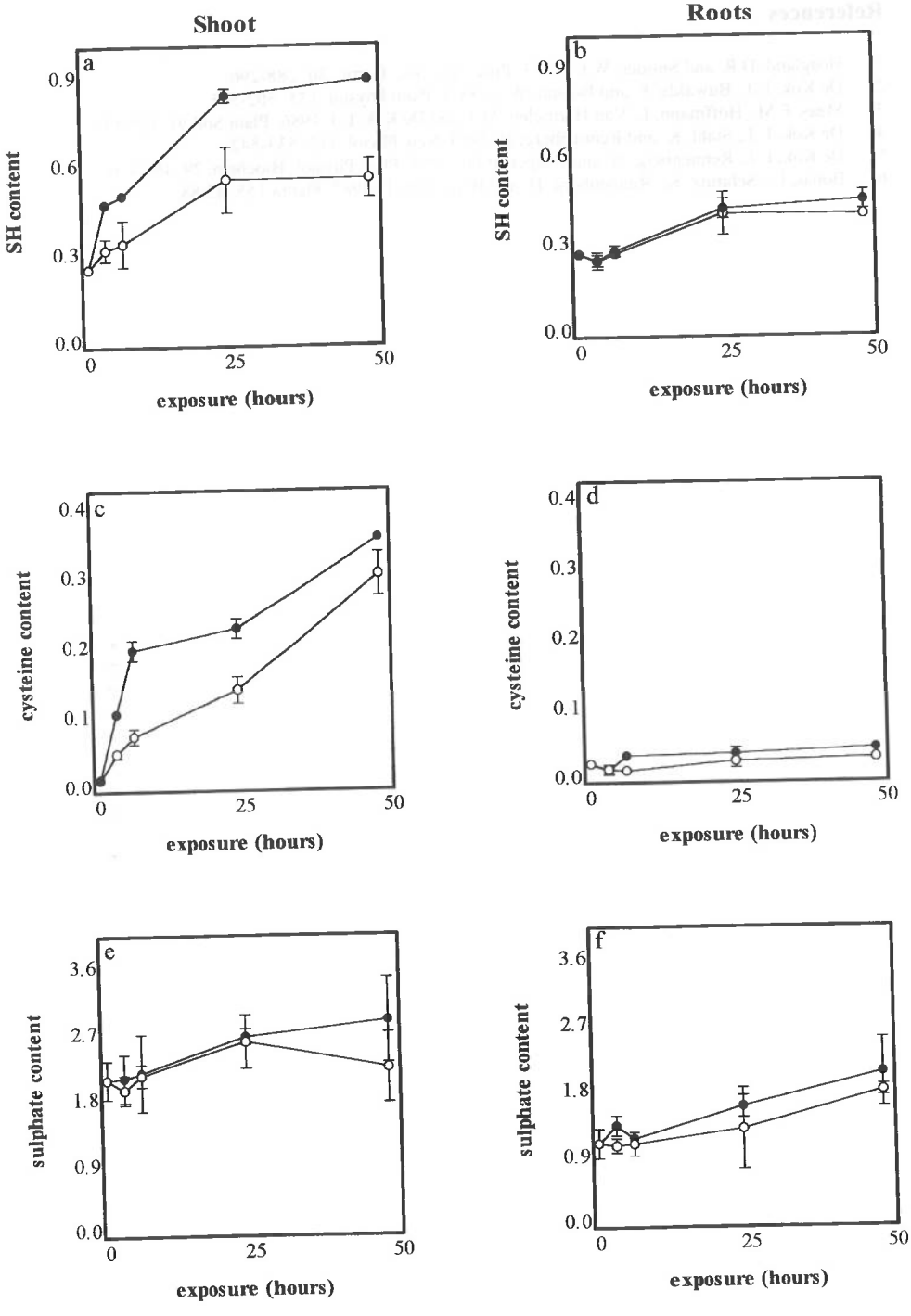


*Fig. 2.* Effect of $H_2S$ exposure on the levels of water-soluble non-protein thiols in shoot (a) and roots (b), cysteine in shoot (c) and roots (d), and sulphate in shoot (e) and roots (f). Plants were exposed to 0.06 (○—○) and 0.3 µl $l^{-1}$ $H_2S$ (●—●). Data are expressed as µmol $g^{-1}$ fresh weight and represent the mean of three independent measurements with three plants in each (±SD).

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# LONG TERM EFFECTS OF NATURALLY ELEVATED $CO_2$, $H_2S$ AND $SO_2$ ON SULPHUR ALLOCATION IN *QUERCUS*

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## Abstract

The thiol composition and contents were analysed in oak trees growing at sites with naturally elevated atmospheric $CO_2$ and sulphur concentrations. Exposure to these gases leads to enhanced thiol contents in metabolically active tissues such as leaves and bark. In both tissues GSH was the predominant thiol. The additional reduced sulphur in leaves and bark seems to originate from atmospheric sulphur influx, since sulphur contents of the xylem sap remained unchanged. In contrast to xylem sap, GSH contents of phloem exudates decreased at elevated compared to ambient $CO_2$ and sulphur independent of the site studied. Enhanced thiol contents in the bark, but reduced thiol contents in the phloem under elevated $CO_2$ and sulphur indicate that reduced sulphur from atmospheric influx is partially stored in the bark and may not be allocated to the root.

Plants are exposed in their environment to a wide range of concentrations of atmospheric sulphur compounds, including sulphur dioxide ($SO_2$) and hydrogen sulphide ($H_2S$). It has been suggested that negative effects on slow growing plants can already be expected if long-term mean concentrations of 2-5 ppb $SO_2$ are exceeded[1,2]. Usually this mean concentration is exceeded by far for both $SO_2$ and $H_2S$ in the vicinity of $CO_2$ springs. On the other hand, enhanced growth and biomass accumulation under elevated $CO_2$[3] requires enhanced reduced sulphur for protein synthesis of growing tissues. Therefore, a rapid flux of $SO_2$ and $H_2S$ into cellular sulphur pools may support growth and development under these conditions. The present experiments were performed to address the question as to whether atmospheric sulphur influx into plants enhances the availability of reduced sulphur for protein synthesis in growth and development in plants exposed to elevated $CO_2$.

The investigations were performed with mature *Quercus pubescens* trees at two different sites in Italy with naturally elevated $CO_2$ and atmospheric sulphur, i.e. Bossoleto (mean of $CO_2$: 1287 ppmv) and Solfatara (mean of $CO_2$: 1146 ppmv), which differed in the atmospheric sulphur gas mixing ratio. In Bossoleto and Solfatara the concentrations of $SO_2$ (mean: 3 ppbv and 160 ppbv, respectively) and $H_2S$ (mean: 7 ppbv and 419 ppbv, respectively) were measured at the centre of the springs. In May/June 1995, samples were collected at each site from trees growing close to the $CO_2$-vent and at a distance where trees experienced ambient $CO_2$ concentrations. Xylem saps were obtained from the branches by the Scholander-technique[4,5], phloem exudates by the EDTA-technique[5]. Leaves and bark were collected and immediately frozen in liquid nitrogen. Sample analysis was carried out as described by Schupp and Rennenberg[6].

Enhanced sulphur content in the close vicinity of the $CO_2$-vents leads to an enhanced concentration of reduced sulphur in leaves and bark of oak trees (Fig. 1). In both tissues GSH was the predominant thiol (50 to 600 nmol $g^{-1}$ FW). Its cellular content increased with increasing sulphur content of the atmosphere independent of the site studied. This

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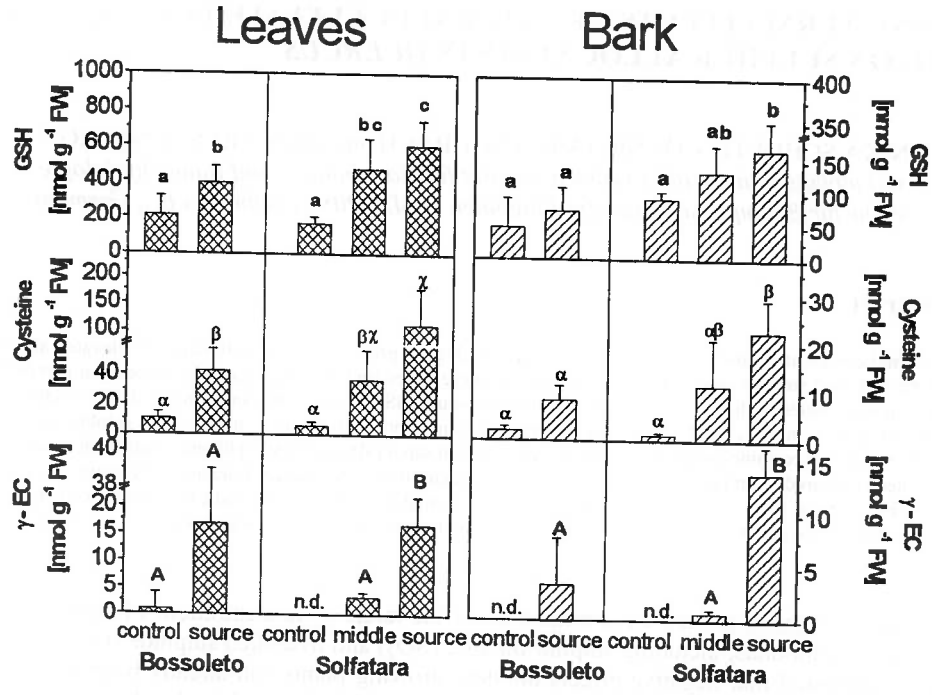


*Fig. 1.* Thiol composition and contents in leaves and bark of *Q. pubescens.* Leaves and bark were collected from the $CO_2$ vent and the control site with ambient $CO_2$. Samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis. Thiols were analysed as described by Schupp and Rennenberg[6].

was also observed for cysteine and γ-glutamylcysteine (γ-EC). In leaves, 4-fold higher concentrations of GSH and cysteine were found as compared to the bark. The concentration of both GSH and cysteine in leaves as well as in the bark were generally higher in Solfatara – the site with high S-pollution even at the control site with ambient $CO_2$ – than in Bossoleto (Fig. 1).

GSH was the most abundant thiol in phloem exudates of *Q. pubescens,* as well as in leaves and bark (Fig. 2). Concentrations of GSH in phloem exudates were lower in trees from elevated than from ambient $CO_2$ sites, but generally much higher in Solfatara. Phloem exudates contained relatively high amounts of cysteine at Bossoleto and much lower amounts of cysteine at Solfatara. In contrast to phloem exudates thiol contents of xylem saps were similar at both sites. Changes in xylem sap sulphur contents at elevated as compared to ambient $CO_2$ were not observed. Whereas at Bossoleto GSH was found to be the dominating thiol in the xylem saps under elevated and ambient $CO_2$, almost equal GSH and cysteine contents were determined at Solfatara independently of the $CO_2$/sulphur exposure (Fig. 2).

Enhanced sulphur content in the close vicinity of $CO_2$-vents lead to an enhanced availability of reduced sulphur for growth and development in metabolic active tissues such as leaves and bark of oak trees. This extra reduced sulphur seems to originate from uptake of atmospheric, but not of pedospheric sulphur, since sulphur concentrations in xylem saps were unchanged. Despite enhanced reduced sulphur contents in bark, sulphur supply from the leaves to the roots by phloem transport is diminished in trees growing close to the vent. Apparently, reduced sulphur from atmospheric influx is partially stored

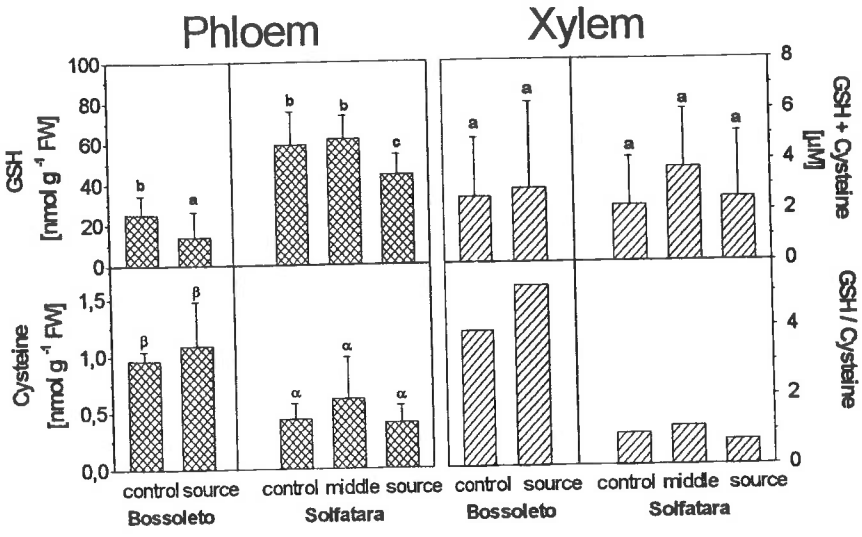


*Fig. 2.* Thiol composition and contents in phloem and xylem of *Q. pubescens.* Xylem sap and phloem exudates were collected from the $CO_2$ vent and the control site with ambient $CO_2$. Samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis. Aliquots of 100 µl (xylem sap) and 400 µl (phloem exudates) were used for analyzing soluble thiols. Thiols were reduced with DTT, derivatized with mBBr and separated by HPLC using the method described by Schupp and Rennenberg[6].

in the bark. Studies of Körner and Miglietta[7] showed that enhanced availability of $CO_2$ does not result in enhanced stem growth. It can therefore be assumed that (1) photosynthesis is down-regulated under continuous exposure of oaks to elevated $CO_2$ and that this down-regulation is not modulated by S availability, or that (2) high atmospheric sulphur contents counteract positive effects of elevated $CO_2$. First measurements of actual photosynthesis seem to support the latter assumption.

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# ATMOSPHERIC $H_2S$ AS SULPHUR SOURCE FOR SULPHUR DEPRIVED *BRASSICA OLERACEA* L. AND *HORDEUM VULGARE* L.

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## Abstract

Curly kale (Brassica oleracea L. cv. Bornick) and spring barley (Hordeum vulgare L. cv. Triumph) were cultivated on a 25 % Hoagland nutrient solution without sulphate. After 1 to 2 weeks of sulphur deprivation plants became sulphur deficient, characterized by a reduced growth, yellowing of the leaves, very low sulphate and thiol contents, and enhanced levels of nitrate and amino acids. Exposure of the sulphur deprived plants to 0.25 $\mu l\ l^{-1}$ $H_2S$ resulted in recovery from sulphur deficiency. Growth was largely restored, and the contents of the various metabolites nearly returned to that in sulphur sufficient plants. Apparently, $H_2S$ can be utilized as sole sulphur source for plant growth.

The impact of sulphurous air pollutants on plants is paradoxical. Chronically high levels of e.g. $SO_2$, or $H_2S$, may negatively affect plant growth, and acute levels may result in visible injury. However, there is a wide variation in susceptibility between species[2]. On the other hand, atmospheric sulphur may directly be utilized as sulphur source for plant growth and it may be beneficial, especially when the sulphur supply to the roots is limited[2]. Plant shoots form a sink for atmospheric $H_2S$, and its uptake is determined by physiological rather than by physical/chemical processes[1,2,3,4]. $H_2S$ is directly incorporated into cysteine and subsequently into other organic sulphur compounds[1,2,3,4].

Curly kale and spring barley, plant species with a high and low sulphur requirement, respectively, were cultivated on a 25% Hoagland nutrient solution with and without sulphate. After 1 to 2 weeks of sulphur deprivation plants became sulphur deficient, and were characterized by: a reduced growth, an enhanced dry matter content (mainly due to enhanced non-structural carbohydrate levels), a decreased pigment content, very low sulphate and thiol contents, and enhanced levels of nitrate and free amino acids (Table 1). Both curly kale and barley appeared not to be susceptible to $H_2S$, as growth of plants at a normal sulphur supply was not significantly affected upon exposure to 0.25 $\mu l\ l^{-1}$ $H_2S$. At a normal sulphur supply the thiol content was considerably increased upon $H_2S$ exposure, as has been observed previously[1,2,3,4], whereas the levels of the other measured metabolites were hardly affected (Table 1). In contrast to curly kale, barley accumulated significant amounts of sulphate upon exposure to $H_2S$. The sulphur deprived plants almost completely recovered from sulphur deficiency after $H_2S$ exposure: growth was largely restored, the shoots regreened, and the dry matter content and the contents of soluble sugars, starch, nitrate and amino acids decreased to nearly control levels. In curly kale the sulphate content remained rather low, whereas in barley it largely returned to levels observed in plants grown at a normal sulphur supply (Table 1).

The present data demonstrate that atmospheric $H_2S$ can be used as sole sulphur source for plant growth.

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*Table 1.* Atmospheric $H_2S$ as sulphur source for curly kale (*Brassica oleraceae* L. cv. Bornick) and spring barley (*Hordeum vulgare* L. cv. Triumph). Seedlings of curly kale and barley were grown on a 25% Hoagland nutrient solution with 0.5 mM sulphate (+S) or 0 mM sulphate (-S; all sulphate salts were replaced by chloride salts) for 14 and 4 days, respectively. Thereafter, the plants were transferred to 1.1 l containers (3 and 4 plants per container for curly kale and barley, respectively) on freshly prepared nutrient solutions (see above), and exposed to 0.25 µl $l^{-1}$ $H_2S$ in fumigation cabinets for 7 (*B. oleracea*) or 8 days (*H. vulgare*). Temperature was 20°C, relative humidity 50-60% and photon fluence rate 180 µmol $m^{-2}$ $s^{-1}$ (within the 400-700 nm range) at a photoperiod of 14 h. The apparent relative growth rate (RGR) on a fresh weight basis was calculated over the 7 or 8 days time interval. Contents of chlorophyll, soluble sugars, starch, sulphate, water-soluble non-protein thiols, nitrate and amino acids were determined as described before[5]. RGR is expressed as %, dry matter content (DMC) as % and chlorophyll as mg $g^{-1}$ fresh weight; the other data are expressed as µmol $g^{-1}$ fresh weight. Different letters indicate significant differences at $p < 0.05$ between sulphur nutrition levels

| | Shoot | | | | Root | | | |
|---|---|---|---|---|---|---|---|---|
| | control | | $H_2S$ | | control | | $H_2S$ | |
| | +S | -S | +S | -S | +S | -S | +S | -S |
| *Brassica oleracea* L. | | | | | | | | |
| RGR | 11 | 6 | 13 | 14 | 10 | 4 | 11 | 11 |
| DMC | 9.5[a] | 14.0[b] | 9.2[a] | 9.2[a] | 7.0[a] | 7.6[a] | 6.5[a] | 6.2[a] |
| Chlorophyll | 1.10[a] | 0.41[c] | 1.20[a] | 0.74[b] | – | – | – | – |
| Soluble sugars | 19[a] | 94[b] | 22[a] | 28[a] | 7[a] | 11[b] | 8[a] | 7[a] |
| Starch | 26[a] | 130[b] | 25[a] | 30[a] | 27[a] | 37[b] | 28[a] | 26[a] |
| Sulphate | 45.3[a] | 0.7[c] | 39.1[a] | 3.1[b] | 6.9[a] | 0.4[b] | 7.8[a] | 0.6[b] |
| Thiols | 0.41[a] | 0.05[d] | 1.18[c] | 0.69[b] | 0.52[a] | 0.13[d] | 0.68[c] | 0.30[b] |
| Nitrate | 162[a] | 238[b] | 152[a] | 160[a] | 34[a] | 66[b] | 38[a] | 47[a] |
| Amino acids | 7.7[a] | 36.9[b] | 8.3[a] | 8.0[a] | 4.2[a] | 9.2[b] | 4.8[a] | 3.6[a] |
| *Hordeum vulgare* L. | | | | | | | | |
| RGR | 14 | 6 | 17 | 12 | 13 | 9 | 17 | 15 |
| DMC | 8.6[a] | 8.9[a] | 9.6[a] | 9.1[a] | 6.7[a] | 9.1[b] | 6.5[a] | 6.8[a] |
| Chlorophyll | 0.92[a] | 0.52[b] | 0.90[a] | 0.89[a] | – | – | – | – |
| Sulphate | 2.06[a] | <0.1[c] | 6.22[d] | 3.13[b] | 3.40[a] | <0.1[d] | 5.51[b] | 0.72[c] |
| Thiols | 0.17[a] | 0.05[b] | 0.52[c] | 0.46[c] | 0.13[a] | 0.06[b] | 0.26[c] | 0.23[c] |
| Nitrate | 69[a] | 90[b] | 75[a] | 65[a] | 79[a] | 113[b] | 79[a] | 88[a] |
| Amino acids | 10.1[a] | 36.0[b] | 13.6[a] | 13.1[a] | 7.8[a] | 59.2[b] | 7.8[a] | 7.9[a] |

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# TRANSGENIC MAIZE WITH ELEVATED 10 KD ZEIN AND METHIONINE

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## Abstract

Kernels of transgenic maize plants with introduced copies of a high methionine 10 kD zein gene were evaluated for 10 kD zein and methionine content. A bar gene was used as a selectable marker. Elevated levels of 10 kD zein and methionine were observed in bar$^{(+)}$ kernels of transformed plants, demonstrating that seeds from maize plants transformed with a gene encoding the 10 kD zein overexpress and accumulate high levels of 10 kD zein and may contain elevated levels of methionine.

Genetic engineering of plants offers considerable promise to modern agriculture and plant breeding. Increased crop value due to enhanced nutritional quality is one of the potential benefits. Enhanced nutritional quality may include grain with improved amino acid composition for livestock feed. To be nutritionally adequate (and to support optimal growth of chickens), poultry feed consisting of maize/soybean (*Zea mays/Glycine max*) meal is typically supplemented with synthetic methionine, and the development of maize lines which supply higher levels of methionine than normal could reduce the need for methionine supplements. Such high methionine maize lines may be generated by introducing into the maize genome a highly expressed gene encoding a methionine-rich protein. The 10 kD zein storage protein of maize kernels is notably high in methionine content (22.5%), and the gene encoding this protein has been cloned and its expression characterized[1,2]. This gene, under the control of endogenous promoters, was reintroduced into the corn genome. The bacterial *bar* gene, which confers resistance to the herbicides bialaphos and glufosinate, was used as a selectable marker[3]. Transformation and regeneration were carried out according to the procedure of Gordon-Kamm *et al*[4], using embryogenic suspension cultures. Transgenic lines were identified by the ability to grow in the presence of the herbicide bialaphos. Calli carrying introduced 10 kD zein genes were identified by polymerase chain reaction (PCR), regenerated and brought to seed.

## Identification of bar$^{(+)}$ and bar$^{(-)}$ kernels

Bar$^{(+)}$ and bar$^{(-)}$ kernels from individual ears segregating for introduced genes were identified by germination of half kernels (with embryos) in the presence of glufosinate or by PCR. The remaining half kernels were analyzed for 10 kD zein and methionine content. Those kernels possessing the bar gene germinated and grew normally in the presence of

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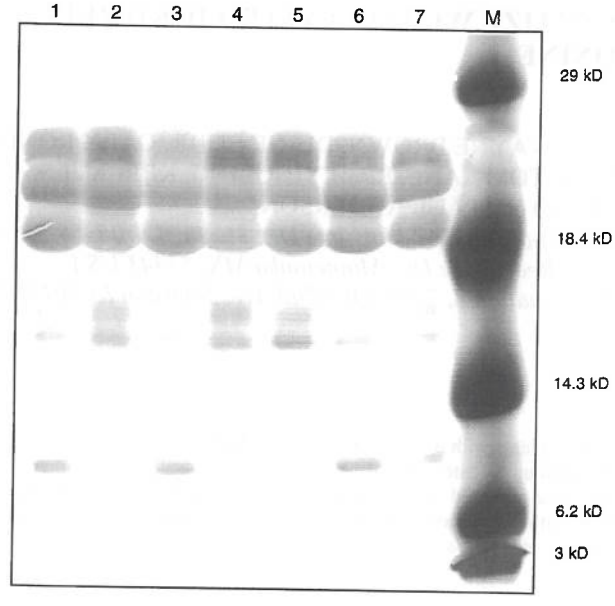


**Segregation of elevated 10 kD zein expression in kernels from a single ear of a transformed plant.** SDS-PAGE analysis of zeins extracted from individual kernels of a transformant expressing high levels of 10 kD zein (lowest band).

*Fig.1.* SDS-PAGE analysis showing segregation of elevated 10 kD zein expression in kernels from a single ear of a transformed plant. Lanes 1-7; Zein profiles of individual kernels of a transformant expressing high levels of 10 kD zein (lowest band); Lane 8; Molecular weight markers.

glufosinate. Those not possessing the bar gene germinated but failed to grow. PCR analysis was carried out on selected kernels to confirm the results of the bioassay. Because kernels which did not germinate were sometimes PCR$^{(+)}$ for the presence of the bar gene, those kernels which did not germinate were discarded. Analysis of 10 kD zein and methionine was carried out on pooled bar$^{(+)}$ and pooled bar$^{(-)}$ kernels from the same ear.

## Analysis of transformants

Ground meal samples from bar$^{(+)}$ and bar$^{(-)}$ kernels were analyzed for 10 kD zein and methionine levels by ELISA and HPLC, respectively. Elevated levels of 10 kD zein and/or methionine were observed in bar$^{(+)}$ kernels from 35 of 48 transformants selected. Total protein content, as determined by Kjeldahl analysis, was approximately equal in transformed and untransformed kernels.

## Results

Seed analysis showed elevated expression of 10 kD zein in seed samples of bar$^{(+)}$ kernels from 28 transformants tested in this experiment, as compared to untransformed kernels from the same ear. A wide range of 10 kD zein levels was observed for selected transformants, from 0% to 0.9%. Methionine levels from kernels of these transformants was as high as 0.63%, representing an increase of approximately 30% in this transfor-

mant (untransformed kernels from the same ear had a methionine content of 0.49%). In some cases, not all crosses from a given transformant showed elevated 10 kD zein. Samples with genomic backgrounds naturally high in 10 kD zein were less likely to show 10 kD zein elevation in bar$^{(+)}$ kernels since elevation in 10 kD zein was easier to detect in low 10 kD zein backgrounds. Elevated methionine content was detected in bar$^{(+)}$ kernels from 19 transformants. Not all transformants with elevated 10 kD zein levels showed a corresponding increase in methionine, and some transformants with elevated kernel methionine did not overexpress 10 kD zein.

Single kernel SDS-PAGE analysis of zein extracts from populations segregating for elevated 10 kD zein (as shown by ELISA) clearly showed an increase in transformed kernels (Fig.1). In some transformants, levels of other zeins remain constant, while in others (such as the transformant shown) a reduction in the 15 and 27 kD zeins, which are also high in sulphur, was also evident.

Our data show that seeds from maize plants transformed with a gene encoding the 10 kD zein overexpress and accumulate high levels of 10 kD zein. These seeds may also contain elevated levels of methionine.

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# REGULATION OF SOYBEAN SEED STORAGE PROTEIN GENES -A MODEL FOR SULPHUR NUTRITION-REGULATED GENE EXPRESSION

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The genes encoding β-conglycinins, major seed storage proteins of soybean (*Glycine max* (L) Merr), are differentially regulated depending on sulphur availability in the environment. Such patterns of regulation were conserved in transgenic petunia and *Arabidopsis thaliana*, suggesting that higher plants share common mechanisms of regulation. Possible mutants of *Arabidopsis* that affect the patterns of the sulphur-regulated expression of the β subunit gene were isolated.

The genes encoding the subunits of β-conglycinin, a major seed storage protein of soybean, are known to differentially respond to sulphur nutritional condition[1]. β-Conglycinin is comprised of three subunits, α, α', and β[2]. Among the subunits, the α' subunit exhibits little or no response to sulphur nutritional conditions, whereas the β subunit is upregulated by sulphur deficiency and repressed by exogenous application of methionine[3,4]. It is established that the β subunit has a very low content of sulphur-containing amino acids, whereas the α and α' subunits have moderate contents[5]. Glycinin, the other major component of soybean seed storage protein, has high S-containing amino acids, and its accumulation is repressed by sulphur deficiency and enhanced by exogenous application of methionine[3,5]. Seed storage proteins are a reservoir of nitrogen and sulphur to be consumed during germination and the phenomena described above are beneficial for plants to conserve as much nutrients as possible depending on the sulphur availability in the environment. Such alteration in the patterns of seed storage protein accumulation have been described in a number of plant species[6]. Our studies have focused on the mechanisms underlying the regulation of the β subunit gene by sulphur nutritional conditions, as a model system for understanding sulphur nutrition-regulated gene expression in higher plants. After finding that the genes encoding the α' and β subunits of β-conglycinin were regulated by sulphur nutrition in transgenic petunia and *Arabidopsis* in similar ways as in soybean[6,7], *Arabidopsis thaliana* was used for our molecular biological and genetical analysis of the process.

We constructed chimeric genes that carried promoter sequences of the α' and β subunit genes upstream of the open reading frame of the *E. coli* β-glucuronidase (GUS) and introduced to *A. thaliana*. Transgenic plants were then exposed to various levels of sulphur deficiency by hydroponic cultures and to exogenously applied methionine. GUS

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activities of mature seeds from treated plants were determined. The GUS activities driven by the β subunit promoter were several fold higher under sulphur deficient conditions than under normal conditions[8] (Fig. 1). The α' subunit promoter did not show striking differences regardless of the sulphate conditions[8]. Methionine application had similar effects in that the expression of the β subunit promoter was repressed, whereas the α' subunit promoter was little affected[9]. The effects of sulphur nutrition were evident throughout the course of seed development and the response of the β subunit genes to sulphur nutritional conditions was reversible, i.e. changes in seed GUS activities were evident in two days after the change of sulphate concentration in the nutrient solution[9]. Such reversibility was also observed in the case of the response to methionine in transgenic *Arabidopsis*[8].

The response of the β subunit can be considered as a part of adaptation response of soybean to accumulate as much nitrogen and sulphur as seed storage proteins. If so, it is likely that the β subunit promoter responds not only to sulphur nutrition but also nitrogen nutrition. The above mentioned transgenic *Arabidopsis* line carrying the β promoter-GUS fusion gene were cultured in various concentration of nitrate supply. GUS activities of mature seeds were reduced as the levels of nitrate were reduced[10], suggesting that the levels of nitrogen also affect the β subunit expression.

To better understand the molecular components involved in this signal transduction pathway of sulphur nutrition regulated gene expression, we undertook genetic approaches. Mutants of *A. thaliana* defective in response to sulphur nutritional conditions were screened. Four approaches have been taken. 1) Selection of selenate resistant mutants; 2) Selection of ethionine resistant mutants; 3) Screening of plant lines that cannot grow under low sulphur supply; 4) Screening of lines defective in regulating the β subunit promoter.

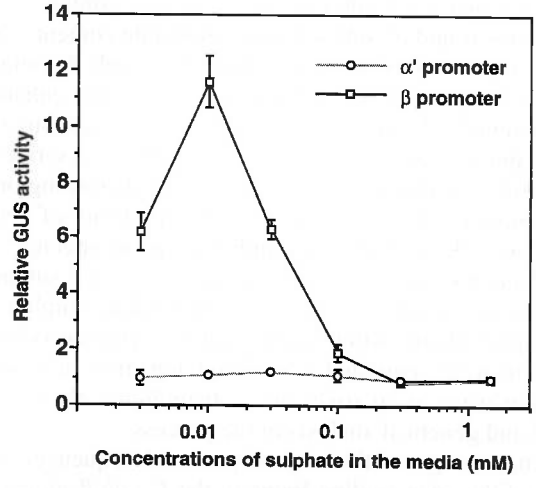


*Fig. 1.* Response of the β-conglycinin promoters to sulphate concentrations in transgenic *A. thaliana*. Transgenic plants carrying either the α' or β subunit gene promoter were cultured hydroponically in the presence of various concentration of sulphate. GUS activities of the mature seeds were measured. GUS activities relative to that in plants grown at 1.5 mM sulphate were plotted.

For the first approach, about 50,000 ethyl methane sulphonate (EMS) treated M2 seeds were germinated on the plates containing 200 μM selenate and no sulphate. On these plates, wild type seeds produced roots up to a few mm long after several days but never expanded cotyledons. We isolated 9 lines of *Arabidopsis* that can expand cotyledons under the selective conditions. Resistance to selenate was inherited in at least four subsequent generations and some of these lines exhibited a reduced rate of sulphate uptake. However, selenate resistance did not seem to be associated with reduced sulphate uptake and the phenotype of reduced sulphate uptake was not stable[11]. We are in the process of re-evaluating the phenotypes related to sulphur assimilation of the selenate resistant lines.

The second approach utilized ethionine, an analogue of methionine. By screening EMS mutagenized M2 seeds, we found several lines of *Arabidopsis* resistant to toxic levels of ethionine. The mutant, termed *mto1-1* (methionine overaccumulation), accumulated nearly 50 fold higher levels of methionine in rosette leaves[12]. Expression of the β subunit promoter in the *mto1-1* background was repressed and tissue specific patterns of expression were also altered[13]. Effects of *mto1-1* were evident only when the mutation was present maternally[13].

Our third approach is based on the assumption that higher plants carry mechanisms to adapt to low concentrations of sulphate. Mutants that have defects in mechanisms of such adaptation would grow very poorly at low sulphur supply compared to wild type plants but grow normally when sulphur was sufficiently supplied. EMS treated M2 seeds of *Arabidopsis* were sown on rockwool and supplied with low concentrations of sulphur (10-30 μM). At this concentration of sulphur supply, wild type plants exhibit very mild symptoms of sulphur deficiency. After three weeks, plants that grew normally were discarded and nutrient solution containing 3 mM sulphate was supplied to the poorly grown plants. Seeds were harvested from recovered plants and subjected to the second round of screening. We currently obtained two putative mutant lines that grow normally at normal concentration of sulphate (1.5 mM) but fail to grow well under sulphur deficient conditions.

The final approach used the expression of a transgene as a screening criterion. As described previously, the β subunit promoter is upregulated by sulphur deficiency. We mutagenized by EMS a line of transgenic *Arabidopsis* carrying multiple copies of the β promoter-GUS fusion gene. By measuring GUS activities of cotyledons of about 20,000 individual seedlings, we identified about 200 candidate lines that exhibited an altered pattern of GUS activity. After subsequent rounds of testing the candidates, we identified a line (146C7h) that respond poorly to sulphur deficiency in terms of upregulation of the β subunit gene (Fig. 2). We are currently in the process of evaluating the nature of the mutation carried by the line.

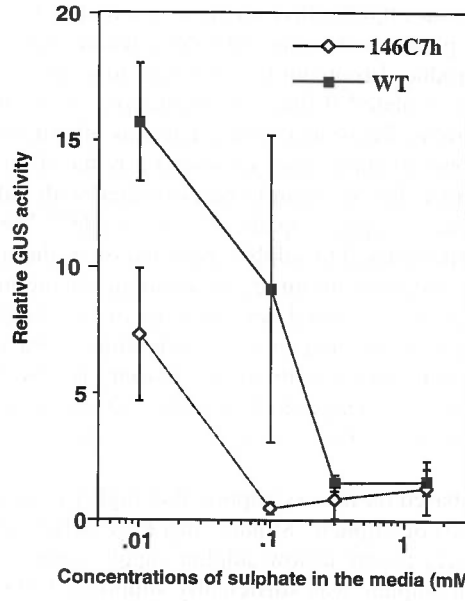


*Fig. 2.* A putative mutant line of *Arabidopsis* that is unable to properly up-regulate the β subunit promoter in response to sulphur deficiency. The original transgenic line (WT) carrying multiple copies of pβ-GUS fusion gene and a line (146C7h) derived from a mutagenized population of the original transgenic line were hydroponically cultured in the media containing various concentration of sulphate. GUS activities (n=5) of mature seeds were determined and GUS activities relative to the control (1.5 mM sulphate) were plotted.

## Acknowledgements

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# EFFECTS OF NITROGEN NUTRITION ON THE EXPRESSION OF A SOYBEAN SEED STORAGE PROTEIN GENE IN TRANSGENIC *ARABIDOPSIS THALIANA*

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## Abstract

The gene encoding the ß subunit of ß-conglycinin, one of the major seed storage proteins of soybean, is known to be upregulated by sulphur deficiency. Transgenic *Arabidopsis thaliana* (L) Heynh. carrying the GUS gene fused to the 5' upstream region of the ß subunit gene was grown by hydroponic culture at various concentrations of nitrate. GUS activities in mature seeds showed positive correlation to nitrate concentrations in the media within a range of 0.10 to 5.0 mM. When ammonium was added to culture media with low nitrate concentration, GUS activities were elevated. These results suggest that the ß subunit gene promoter is regulated by nitrogen nutrition in addition to sulphur nutrition.

Accumulation of β subunit in seeds increases in sulphur-deficient soybean plants[1]. We previously showed that expression of the β subunit gene is upregulated by sulphur deficiency mainly at the level of transcription in transgenic plants[2,3]. As a number of studies suggested interaction of sulphur and nitrogen nutrition[4], we investigated whether the β subunit gene is also regulated by nitrogen nutrition or not.

A line of transgenic *A. thaliana* which carries the β subunit gene promoter-GUS chimeric gene, named *SNTβ39*[5], was grown by hydroponic culture as described previously[3] at 0.10, 0.33, 1.0, 5.0 and 15 mM nitrate. Nitrate is a sole nitrogen source of the culture medium used in this study[3]. GUS activities of mature seeds were measured. Within the range of 0.10 to 5.0 mM, GUS activities became higher as nitrate concentrations became higher (Fig. 1). When transgenic *A. thaliana* carrying the GUS gene fused to the 5' upstream region of the gene encoding *α'* subunit of β-conglycinin, which does not respond to sulphur deficiency[1,3], was cultured at various concentrations of nitrate, GUS activities did not show significant correlation to nitrate concentration (data not shown). At 15 mM, GUS activity became lower (Fig. 1). We consider this may be due to reduction of transcriptional activities of almost all genes under nitrogen-excess condition.

In order to investigate whether the β subunit gene promoter responds only to nitrate or to nitrogen nutrition, ammonium was added to culture media with low nitrate concentration (0.33 mM, 1.0 mM). Application of ammonium caused GUS activities to become higher in both cases (Fig. 2), suggesting that the β subunit gene promoter also responds to ammonium.

We conclude that expression of the β subunit gene is also regulated by nitrogen nutrition at the level of transcription.

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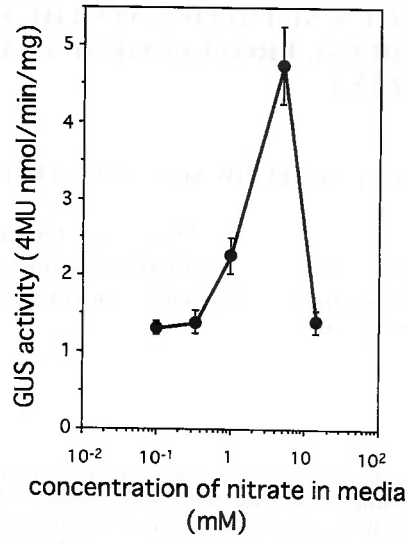


*Fig.1.* Expression of the β subunit gene promoter-GUS chimeric gene in response to nitrate in culture media. Transgenic *A. thaliana* was grown by hydroponic culture at 22°C under natural light. Culture media contained 0.10 to 15 mM nitrate and 1.5 mM sulphate. GUS activities of mature seeds were measured using 4-methylumbelliferyl-β-D-glucuronide as a substrate[6]. The amount of total seed proteins was measured according to Bradford[7]. Each point represents the average ± SE of 3 to 9 samples. Student's *t* test indicated that the GUS activity at 5.0 mM was significantly different from that at other concentrations ($P<0.02$). The GUS activity at 1.0 mM was also significantly different from that at other concentrations ($P<0.02$).

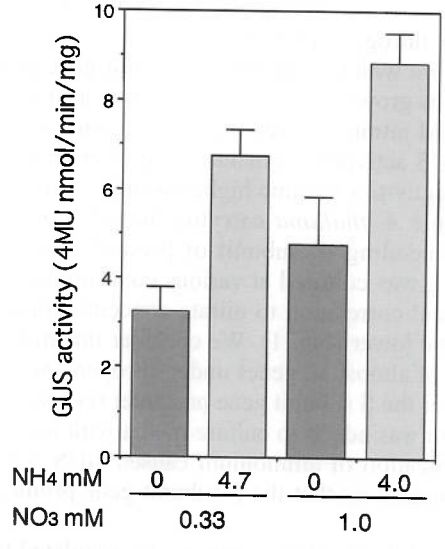


*Fig. 2.* Expression of the β subunit gene promoter-GUS chimeric gene in response to ammonium in culture media. Growth conditions were described in Fig. 1. 4.7 and 4.0 mM ammonium chloride were added to culture media containing 0.33 and 1.0 mM nitrate, respectively, to raise total nitrogen concentration to 5.0 mM. GUS activities of mature seeds were measured as described in Fig. 1. Each bar represents the average ± SE of 10 samples. Student's *t* test indicated that addition of ammonium caused significant differences in GUS activities ($P<0.01$) at both concentrations of nitrate.

## Acknowledgments

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# ANALYSIS OF *O*-ACETYL-L-SERINE IN *IN VITRO* CULTURED SOYBEAN COTYLEDONS

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## Abstract

In this study, we developed a simple method of measuring the concentration of *O*-acetyl-L-serine (OAS) in soybean cotyledons. OAS was separated by the HPLC with a modified buffer for amino acid analysis and detected after reaction with *O*-phthalaldehyde (OPA). Standard OAS was detected as a single peak at 29 min. OAS was also detected in extracts from soybean cotyledons cultured *in vitro*. The concentration of OAS was higher in cotyledons exposed to sulphur deficiency.

The biosynthesis of L-cysteine plays a central role in the sulphur assimilation pathway. It is known that *O*-acetyl-L-serine (OAS) is one of the substrates for cysteine synthesis in microorganisms and in higher plants[1,2,3,4]. A regulatory effect of OAS on the pathway have also been suggested[5,6,7]. To study the regulatory role of OAS in plants, it was important to measure the cellular concentration of OAS. Here, we describe a quick and simple method to do this.

OAS was analyzed by HPLC (model L-6200: Hitachi, Tokyo, Japan) using 4 mm diameter × 150 mm long column packed with No. 2619 resine (Hitachi, Tokyo, Japan) at 37°C. Standard OAS (*O*-acetyl-L-serine hydrochloride: Sigma, St. Louis, MO) and standard amino acid mixtures (amino acid standard. solution., type H: Wako, Osaka, Japan) were dissolved in 0.01 N HCl and subjected to HPLC analysis with a modified $Li^+$ buffer (0.024 M trilithium citrate tetrahydrate, 0.035 M lithium chloride, 0.113 M citric acid, 2.8 % ethanol, pH adjusted to 2.77 with citrate) and detected by fluorescence spectrophotometry after post-column-reaction with *O*-phthalaldehyde (OPA). Aliquots of standard solutions were stored at -20°C. The peak area corresponding to OAS did not change significantly during storage for up to a few months. A typical profile of standard OAS is shown in Fig. 1a. OAS was detected as a single peak at 29 min. The minor peak at 37 min. was found to be serine, which was a contaminant in standard OAS. When mixed with the standard amino acid mixture, OAS was separated from other amino acids (Fig. 1b). It is known that OAS is unstable at room temperature and that intramolecular acyl group conversion from *O*- to *N*- occurs non-enzymatically at a rate of about 1% per min. at neutral pH[8,9]. Furthermore, the conversion from OAS to *N*- acetyl-L-serine (NAS) occurs more rapidly at alkaline pH[9,10,11]. When standard OAS was adjusted to pH 8 with NaOH, the peak corresponding to OAS disappeared (data not shown). It is known that NAS is not detectable by OPA reaction as the amino residue is blocked by acylation. It is likely that disappearance of the peak corresponding to OAS in alkaline solution reflects interconversion of OAS to NAS, suggesting that our peak identification

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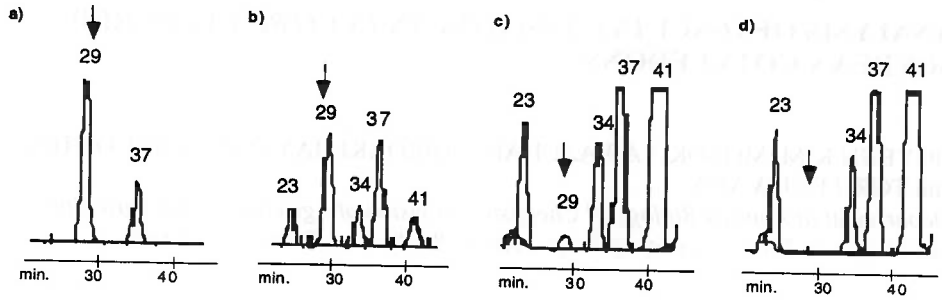


*Fig. 1.* A typical HPLC profiles of OAS analysis. a) OAS standard (1.5 nmol/30 µl). b) The normal standard amino acid mixtures (0.1 nmol/20 µl) mixed with standard OAS (1.0 nmol/20 µl). Peaks represent Asp; 23 min., OAS; 29 min., Thr; 34 min., Ser; 37 min., Asn; 41 min. c) Amino acid extracts from soybean cotyledons dissolved in 0.01 N HCl (6 vol/mg fresh weight). d) Amino acid extracts from soybean cotyledons adjusted to pH 8 with NaOH. The arrow shows the position of the OAS peak (29 min.).

was correct. Fig. 1c shows a profile of amino acid extracted from soybean cotyledons cultured *in vitro* in the presence of 300 µM sulphate. Amino acids were extracted in 80% ethanol at 45°C from soybean cotyledons that had been stored at -80°C. Supernatants were collected and evaporated at room temperature before dissolving in 0.01 N HCl. By HPLC analysis, the OAS peak was detected at 29 min. To confirm the identification of the peak, 0.1 nmol of standard OAS was added to the extracts. The height of the peak at 29 min. increased to the extent equivalent to the amount added (data not shown). Furthermore, when amino acid extracts were adjusted to pH 8, the peak corresponding to OAS disappeared, whereas the peaks of other amino acids did not change (Fig. 1d).

As OAS was suggested to be involved in regulation of the sulphur assimilation pathway, we compared the concentration of OAS in soybean cotyledons cultured in the presence of 300 or 1500 µM sulphate. At 300 µM, the concentration was about 7.3 fold higher than that at 1500 µM (Table 1).

In this study we have developed a method which allows determination of the OAS concentration in plants. Using the method, we have found that OAS is accumulated in soybean cotyledons exposed to sulphur deficiency.

*Table 1.* Concentration of OAS in the soybean cotyledons cultured *in vitro* under sulphur supply (1500 µM) and sulphur deficiency (300 µM). Three measurements were performed for each analysis and mean values and SE from two to four experiments are presented

| Sulphate concentration in the medium (µM) | OAS concentration (nmol/g fresh weight) |
|---|---|
| 300 | 35.7 ± 5.3 |
| 1500 | 4.9 ± 1.0 |

## Acknowledgements

We thank Y. Ishiwatari for many helpful suggestions about our manuscript. This work was supported in part by a grant from The IIJIMA Memorial Foundation for the Promotion of Food Science and Technology ( Japan ) to HYK, a Grant-in-Aid for Scientific Research on Priority Areas ("The Molecular Basis of Flexible Organ Plans in Plants", No. 07270203) to TF and in part by a grant from The Soy Protein Research Committee ( Japan ) to TF.

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# CHANGES IN METALLOTHIONEIN GENE EXPRESSION IN RESPONSE TO SULPHUR NUTRITION IN *cad* MUTANTS

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## Abstract

In *cad1-3* and *cad2-1* mutants of *Arabidopsis thaliana*, which have defects in phytochelatin and glutathione syntheses, respectively, growth under various levels of sulphur supply were not different from wild type. Patterns of expression of *Arabidopsis* metallothionein 2 (MT2) differed between *cad* mutants and wild type. The amount of MT2 mRNA was reduced under sulphur deficiency in *cad1-3* and *cad2-1* while reduction was not evident in the wild type. The degree of reduction is much greater in *cad2-1* than in *cad1-3*.

Sulphur is an essential element for all living organisms including higher plants. When higher plants are exposed to sulphur deficiency, changes in gene expression and metabolic activities occur[1,2,3,4]. These changes may reflect parts of mechanisms to maintain relatively normal growth under limited sulphur supply. The *cad1-3*[5,6] and *cad2-1*[7]mutants of *Arabidopsis thaliana*, which were isolated as cadmium sensitive mutants, have been described as carrying defects in the sulphur assimilation pathway. These mutants are incapable of phytochelatin and glutathione syntheses, respectively[5,7]. It was possible that these mutants show altered response to sulphur deficiency due to the mutations.

We tested the response by two criteria, growth and gene expression. First, we tested the effect of various sulphur nutritional levels on plant growth of these mutants. Columbia wild type and *cad1-3* and *cad2-1* mutants were grown under various levels of sulphur supply. After three weeks of culture, the fresh weight of aerial parts of each plant were measured individually. As shown in Fig.1, both mutants showed no significant difference from wild type in overall growth under various sulphur concentration. Growth at 150 μM $SO_4^{2-}$ were similar to that at 1500 μM $SO_4^{2-}$. This result suggests that defects in these mutants are not located in the pathway of higher plants for adaptation to sulphur deficiency.

To determine the effects of mutations in response to various sulphur levels at the molecular level, we studied patterns of gene expression. We chose the *Arabidopsis* metallothionein 2 (MT2) (K. Takahashi, Laboratory of Plant Nutrition, Department of Agricultural Chemistry Faculty of Agriculture, The University of Tokyo, Tokyo, Japan; GenBank, EMBL, DDBJ accession number X62818) for this analysis. This was because MT2 gene expression was possibly affected by the sulphur level due to its high cysteine content (about 18% of total amino acids[8]). Total RNA was extracted from the aerial portions of *cad1-3*, *cad2-1* and wild type plants grown under various levels of sulphur supply, and subjected to northern analysis. In the wild type, the levels of the MT2 mRNA accumulation were not changed under sulphur deficient conditions. On the other hand, the accumulation of MT2 mRNA was reduced under sulphur deficiency in *cad1-3* and *cad2-1* (Fig.2). The degree of reduction was more evident in *cad2-1* than in *cad1-3*.

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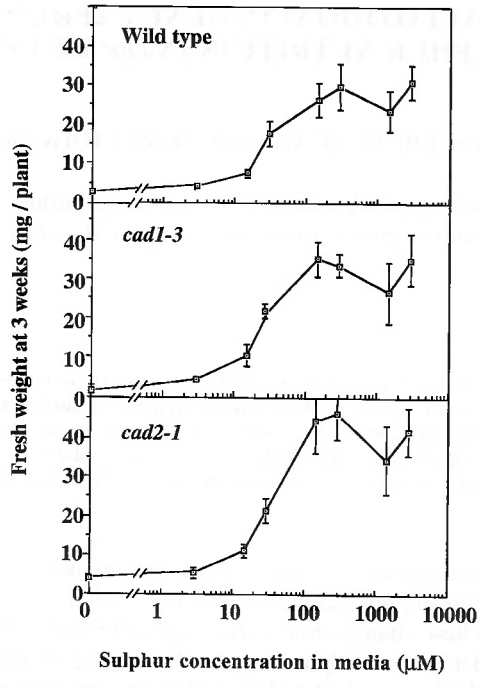


*Fig.1.* Growth of *Arabidopsis thaliana* plants under various sulphur concentration. Growth of Columbia wild type, *cad1-3* and *cad2-1* mutants under various levels of sulphate supply are shown. Plants were grown on rock wool bricks and fed with modified MGRL hydroponic solutions[10] containing 0, 3, 15, 30, 150, 300, 1500 or 3000 μM $SO_4^{2-}$. After three weeks of culture in the greenhouse under natural light, fresh weights of aerial portions of each plant were measured. Average and S.D. (n=18) for each treatment are presented.

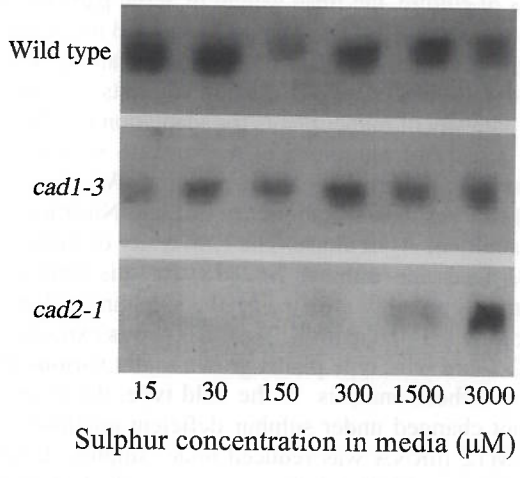


*Fig.2.* MT2 mRNA accumulation under various levels of sulphur concentrations in the media. Plants were grown on rock wool bricks supplied with modified MGRL hydroponic culture media containing 15, 30, 150, 300, 1500 or 3000 μM $SO_4^{2-}$ for three weeks. Aerial parts of plants were harvested and RNA was extracted. Northern analysis was carried out using metallothionein (MT2) cDNA as probes after labelling with fluorescein (ECL™ direct nucleic acid labelling and detection system, Amersham).

These results suggest that expression of the MT2 gene in response to sulphur deficiency was affected by these mutations. However, patterns of expression of the gene encoding ATP-sulphulyrase (ATP-S)[9] were similar among the three lines tested under these conditions (data not shown).

In summary, we found that the expression of the MT2 gene was altered in the mutants defective in phytochelatin biosynthesis, though the growth of mutants was not distinguishable from the wild type plant under various sulphur nutritional levels. To our knowledge, this is the first example of a *trans* mutation which affects patterns of gene expression in response to sulphur deficiency.

## Acknowledgments

We thank to Dr. C. S. Cobbett and Dr. T. Leustek for kindly providing *cad* mutants and providing the cDNA clone of ATP-S, respectively. We also thank Mr. Y. Ishiwatari for critical comments on the manuscript. This work was supported in part by a grant from The Soy Protein Research Committee (Japan) to TF, a Grant-in-Aid for Scientific Research on Priority Areas ("The Molecular Basis of Flexible Organ Plans in Plants", No. 07270203) to TF.

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# SULPHATE CONCENTRATIONS IN WHEAT EPIDERMIS AT SINGLE-CELL RESOLUTION. INFLUENCE OF N & S SUPPLY.

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## Abstract

Wheat was grown in three different hydroponic media containing either 1:8, 1:1 or 8:1 S:N. Solute contents of individual epidermal bulliform cell vacuoles were measured by single cell sampling and analysis techniques (SiCSA[4]). Sulphate accumulated to high concentration in the 8:1 S:N treatment only and was accompanied by a diminished potassium export. Despite a wide range of individual solute concentrations, osmotic pressure was uniform with both time and nutrient treatment.

Following the Clean Air Act of the early 1980s, there is an increasing risk of sulphur deficiency in arable crops across Northern Europe. When supplied to excess, sulphur is readily stored in leaf vacuoles. Remobilisation of S from these vacuoles (even under severe sulphur stress) is however, too slow to support plant growth without a continual supply of external S[1]. Regulation of S remobilisation may occur at the level of the tonoplast[2]. Different cereal leaf cell types accumulate solutes to different levels[3].

A hypothesis was proposed in which the effects of external S supply and S:N ratio on sulphur accumulation in one of these cell types (upper epidermal bulliform trough cells of wheat) could be investigated. *Triticum aestivum* cv Alexandria was grown hydroponically in Long Ashton media containing 1:8, 1:1 or 8:1 S:N (treatments A, B and C respectively). Individual epidermal bulliform vacuoles were sampled with a fine glass microcapillary from the third leaf 2 days before full expansion up to advanced senescence. Osmotic pressure and electrolyte concentrations were determined by picolitre osmometry and X-ray microanalysis[4] respectively.

Epidermal $SO_4^{2-}$ content was negligible (generally less than 10 mM) in low S treatments but accumulated to considerable levels (greater than 150 mM) when N was limiting and S abundant (Fig. 1, treatment C). Total solute content (as osmotic pressure) was unaffected by leaf age or S and N supply (Fig. 2). Epidermal $Cl^-$ content fell below 25 mM with leaf development in all treatments (Fig. 3). The higher $Cl^-$ levels for days -2 to +4 (Fig. 3) represent an experimental artefact from attempts to initially maintain constant medium pH. Epidermal $Ca^{2+}$ accumulated linearly with time during leaf development (Fig. 4). This behaviour was unaffected by sulphate or nitrate status. In the absence of elevated epidermal $SO_4^{2-}$, epidermal $K^+$ declined progressively during leaf development (Fig. 5). This was greatly delayed under conditions inducing high epidermal $SO_4^{2-}$where high $K^+$ levels (ca. 200 mM) remained even under senescent conditions (days 15-26). Tight coupling of $Ca^{2+}$ and $K^+$ concentrations was seen under conditions inducing low epidermal $SO_4^{2-}$. A plot of $Ca^{2+}$ against $K^+$ (Fig. 6) shows a 3:2 replacement of $K^+$ by $Ca^{2+}$ as leaves aged. This ratio is precisely that required if both charge balance and

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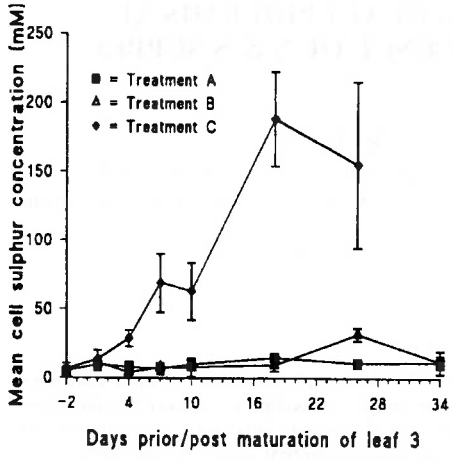


*Fig. 1.* Sulphate concentrations (measured as S) of wheat leaf epidermal bulliform cell vacuoles. Three nutrient regimes were used. Data expressed as means ± SD (n = 45).

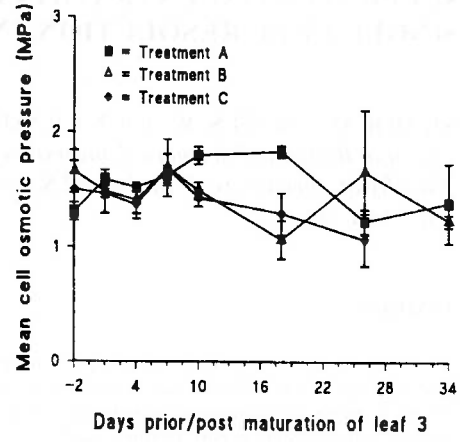


*Fig. 2.* Osmotic pressure of cells in Fig. 1.

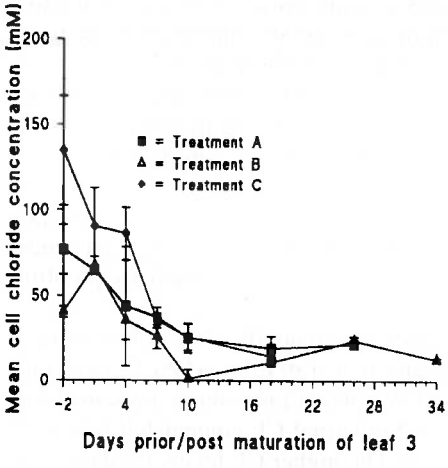


*Fig. 3.* Chloride concentrations of cells in Fig. 1.

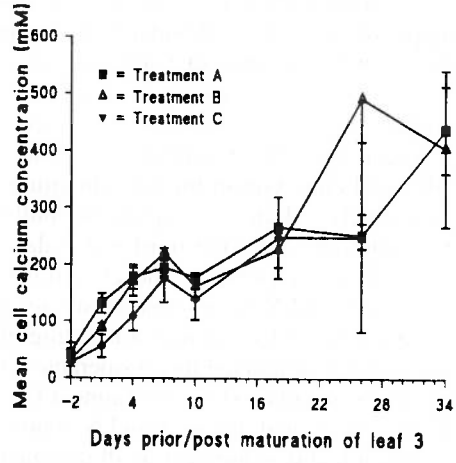


*Fig. 4.* Calcium concentrations of cells in Fig. 1.

osmotic regulation are to be controlled (three $K^+$ and three monovalent anions are replaced by two $Ca^{2+}$ and four monovalent anions). When a divalent anion is available, $Ca^{2+}$-$K^+$ coupling is unnecessary and is lost (Fig. 6, treatment C). A similar situation has been reported for epidermal cells accumulating malate[3].

Sulphate only accumulated in wheat epidermis under low N supply. Despite this, epidermal osmotic pressure was maintained constant both with time and over a wide range of individual solute concentrations. This implies that regulation of osmotic pressure is important but identity of solutes is less so. Clearly the osmotic solution used to generate osmotic and turgor pressure is readily replaced by alternatives. Epidermal osmotic pressure was also constant throughout development (from pre-full expansion to senescence). The influx

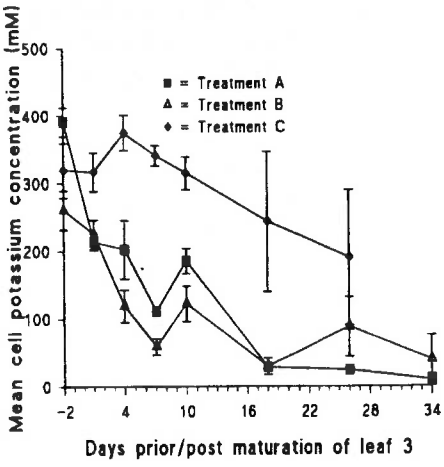


*Fig. 5.* Potassium concentrations of cells in Fig. 1.

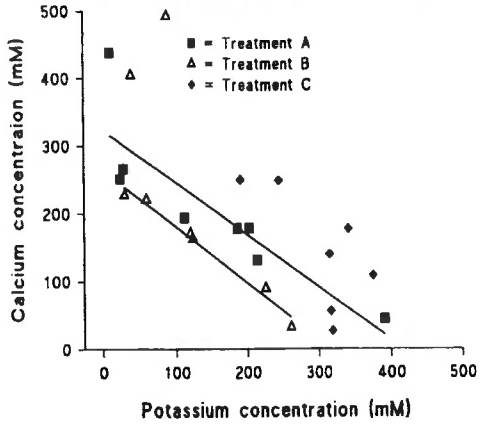


*Fig. 6.* Calcium and potassium concentrations are related in the absence of divalent anions (treatments A and B). The presence of sulphate uncouples this relationship (treatment C).

of solutes such as calcium and sulphate was matched by loss of monovalent electrolytes. Comparison of data for sum of solutes with that for osmotic pressure indicated presence of significant quantities of unidentified anion components (most likely to be $NO_3^-$). Indeed, there appear to be negligible epidermal non-electrolytes. Finally, epidermal accumulation of sulphate is associated with maintenance of high potassium concentration even as the plant senesces. The implications of this will be the subject of future work.

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# TRANSLOCATION OF SULPHUR (S) IN TERRESTRIAL ECOSYSTEMS – A FIELD APPROACH USING STABLE S-ISOTOPE ANALYSIS TO TRACE FERTILISER-S

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## Abstract

$^{34}S$ enriched fertiliser was applied to the soil of an agro-ecosystem. Alteration in S content and S isotopic composition of soil, seepage water and winter wheat was used to get information about S movement in soil and into the plants. After S fertiliser application little increase in total S content and $\delta^{34}S$ values of the soil was observed. Plant available sulphate however increased immediately in the upper soil horizons and S moved into deeper soil layers during the vegetative period. Seepage water reflected fertiliser S only at 30 cm depth. In the plant, all organs except the ears showed an increase in S concentration, but the $\delta^{34}S$ values indicated that fertiliser-S was translocated into all organs including the ears.

Although sulphur is one of the higher plant macronutrients, less attention has been paid to S cycling in agricultural ecosystems than to cycles of the nutrients N, P and K[1].

Input/output mass balances only describe the net results of several processes involved in S-turnover some of which operate in opposite directions. The use of stable isotopes provides a means of tracing nutrient cycles in terrestrial ecosystems under field conditions[3,4]. Provided a difference in the S-isotopic composition of the natural background components and the material applied to the ecosystem exists, tracing of material enriched in one of the stable isotopes is hence possible on a field scale.

The study reported here concerns use of $^{34}S$ to trace S movements in an agro-ecosystem. 50 kg S $ha^{-1}$ $yr^{-1}$ $MgSO_4$ with a $\delta^{34}S$-value of + 9.8‰ was applied to loess with winter wheat grown on it. The fertiliser-S isotopic composition differed by more than 5‰ from the S-isotopic composition of the naturally occurring S components in the research area (mean $\delta^{34}S$-values: rainwater-$SO_4^{2-}$ + 3.16‰; air-$SO_2$ + 1.88‰; plant available $SO_4^{2-}$ in soil + 2.19‰) which allowed the S to be traced through the system.

Soil, seepage water and plant samples were taken weekly from the S treated and a S untreated control plot. Soil samples were taken from 0-10, 10-20, 20-30, 30-60 and 60-90 cm depth and six samples each were bulked in order to give adequate mass for analysis. Plants were separated into all available organs depending on the developmental stage. Total S-content of soil and plants was determined with a S-analyser (Leco SC 132). S-isotope analysis was carried out as described in Giesemann et al.[2].

In the soil fertiliser application led to little increase in the total S content in the upper horizons, while the amount of plant available sulphate (S extracted with 0.016 M $KH_2PO_4$) increased as expected at all soil depths. As the amount of fertiliser-S was only 6% of the naturally occurring S-content, changes in $\delta^{34}S_{total}$-values did not appear. However the alteration of the $\delta^{34}S$-values of plant available sulphate indicated a time dependent translocation of fertiliser-S into the deeper soil layers (Fig. 1).

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The S-isotopic composition of the seepage water collected at 30 cm depth from the fertilised plot showed an increase in $\delta^{34}$S-values immediately after fertiliser application. This showed that fertiliser had been translocated into this soil region. Samples from 60 and 90 cm depths had $\delta^{34}$S-values equal to those from the untreated control plot and hence were not influenced by fertiliser-S.

All plant parts except the ears harvested from the S-treated area showed a slight increase in S-content compared to the control samples. This increase was apparent throughout the whole vegetative period, while the S-content of samples taken from the control plot decreased with time. The $\delta^{34}$S-values proved the observed increase in S-content to be a result of fertiliser uptake into the treated plants. The S-isotopic composition of the ears harvested from the fertilised plot was different from that of the control plot. It approached the $\delta^{34}$S-value of the fertiliser, indicating that most of its S came from the fertiliser, although the S content of ears from both plots was similar (Fig. 2).

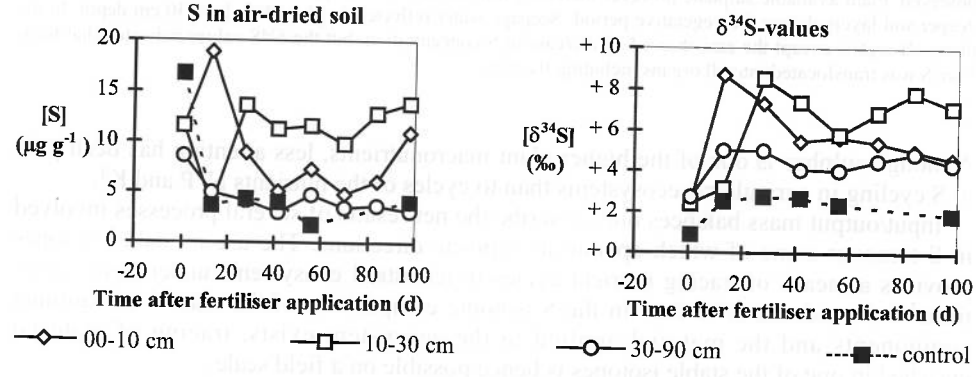


*Fig. 1.* Changes in $S_{plant\ available}$-content and $\delta^{34}S_{plant\ available}$-values in three soil depths (0-10, 10-30 and 30-90 cm) after S-fertilisation of winter wheat under field conditions (fertiliser was applied on the 2nd May 1994). The results from the control plot are shown as means calculated for all soil depths each.

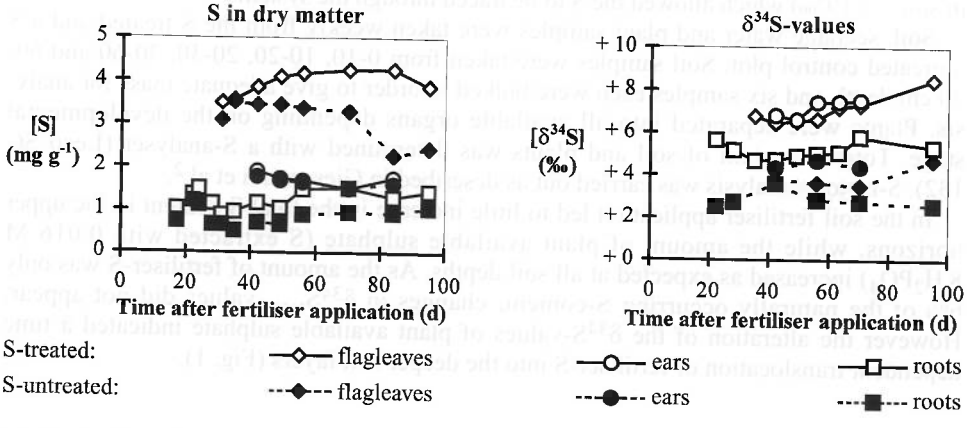


*Fig. 2.* Time dependent changes of the $S_{total}$-content and $\delta^{34}S_{total}$-values in selected plant parts (flag leaves, ears and roots) after S-fertilisation of winter wheat gown under field conditions (fertiliser applied 2 May 1994).

Determination of S-isotopic composition of other S-fractions in soil and plants is currently being carried out. These preliminary results from a field approach to evaluate S-turnover-processes in an agricultural ecosystem by means of stable S-isotope analysis are being further analysed to estimate flows within the system.

## Acknowledgements

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# IMPROVEMENT OF METHIONINE STATUS IN PLANTS UNDER NaCl SALINITY

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## Abstract

The mechanism of methionine (Met) overproduction in salt-resistant cells was studied by comparing the initial wild-type line of Nicotiana sylvestris L. sensitive to NaCl and a mutant salt-resistant line selected under high NaCl concentration (170 mM). The changes in the intracellular pools of amino acids, the end products of the branched pathway of Met biosynthesis, were estimated following the addition of lysine, threonine, and Met to the medium. These changes were interpreted as indicating the regulation of key enzymes involved in Met biosynthesis. It was found that Met overproduction in the salt-resistant cells was apparently due to a decrease in the sensitivity of aspartate kinase (EC 2.7.2.4) to threonine and Met, and in that of cystathionine-γ-synthase (EC 4.2.99.9) to Met.

Abiotic stresses such as soil salinity are the most limiting factors for agricultural productivity and nutritional quality of the yield worldwide[1]. In salt-sensitive species as well as in many salt-tolerant species (halophytes) continuously grown under salinity (NaCl, $Na_2SO_4$), drastic changes in sulphur metabolism are recognized[2]. In particular, excessive sulphate in plant tissues induces oxidative pathways of the conversion of sulphur-containing amino acids[2]. As a result some oxidized Met-derivatives (Met SO, Met $SO_2$, "metabolic" sulphate) are found in plant tissues under sulphate salinity[2]. Another type of change in sulphur metabolism occurs in plants cultivated under NaCl salinity. Under such conditions sulphur deficiency in plants develops. NaCl-sensitive plant species are characterized by a low content of free Met in their tissues[2]. An increased ability of S-deficient plants to convert exogenous Met into cysteine[3] is also observed.

Thus, Met biosynthesis and metabolism are crucial processes for plants grown in environments with NaCl salinity. Such S-deficient plants are usually characterized by low yield as well as low content of essential methionine, while NaCl-resistant, S-sufficient, plants over-produce Met. From a breeding point of view, the plant genotype has to be considered tolerant if it can withstand the stress, but at the cost of commercial yield. The combination of salt tolerance and methionine overproduction opens new possibilities to improve the quality of agricultural crops under extreme conditions of salinity.

To elucidate the mechanism of Met overproduction in a NaCl-resistant cell line a suspension culture of the initial wild-type line of *Nicotiana sylvestris* L., and one of a spontaneous NaCl-resistant mutant line selected under a high NaCl concentration (170 mM) were used. The mutant line was characterized by Met overproduction (Fig.1).

As considered by some authors[4], lysine is one of the main modulators of Met biosynthesis. The results of this work demonstrate that high concentrations of lysine added to Murashige-Skoog medium for cultivation of cell lines do not inhibit aspartate kinase both in NaCl-resistant cells and in NaCl-sensitive cells. One of the reasons for Met overproduction in NaCl-resistant cells could be a disturbance of the feedback regulation of aspar-

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tate kinase by threonine and Met (Table 1). The domination of cysteine over methionine in the cells of the wild-type line and, by contrast, the absence of cysteine in the salt resistant cells points to the insensitivity of cystathionine-γ-synthase for methionine inhibition in the NaCl-resistant cell line (Fig.1). The increase in Met production correlates with the cell line's resistance to chloride. It has been shown earlier that these cells are able to accumulate polyamines under chloride salinity[5]. Taking this into account, we suggest that the plant's capability for Met overproduction not only makes the agricultural products more valuable but also contributes to the adaptation to the excess chloride in the medium.

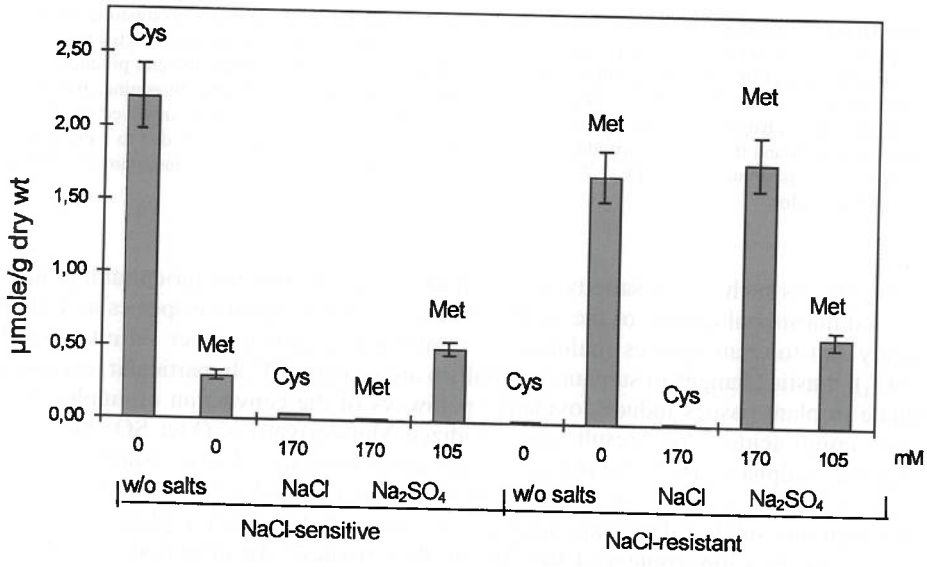


*Fig.1.* Content of free cysteine (Cys) and methionine (Met) in the cells of NaCl-sensitive and NaCl-resistant lines of *Nicotiana sylvestris* L.

*Table 1.* Changes in the content of endogenous lysine in the cells of *Nicotiana sylvestris* L. as affected by exogenous threonine and Met. Suspension cultures of wild-type cells and a mutant NaCl-resistant cell line were grown in M-S medium supplemented with 2,4-D and kinetin[5]. To study the regulation of Met biosynthesis, cell suspensions of both wild-type and mutant line were supplied with L-threonine or L-Met at a concentation of 5mM. At the end of the subculture cycle (14 days), the cells were collected on a glass filter and washed five times with a nutrient medium (without hormones and amino acids). The cells were fixed in liquid nitrogen, freeze-dried, and 0.5-1.0 g of samples were used to estimate the free amino acid content with an AAA-881 analyzer (KOVO, Czechoslovakia). The table presents the means of 3-5 experiments with differences significant at the 0.05 level

| Cell line | Conditions of cultivation | Supplement (5 mM) | Lysine content % of control[a] |
|---|---|---|---|
| NaCl-sensitive (wild-type) | M-S medium without NaCl | Threonine | 12 |
| | | Methionine | 60 |
| NaCl-resistant | M-S medium without NaCl | Threonine | 98 |
| | | Methionine | 430 |
| NaCl-resistant | M-S medium + 170 mM NaCl | Theonine | 103 |
| | | Methionine | 305 |

[a] control: without amino acid addition

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# THE EFFECTIVENESS OF FOLIAR GLUCOSINOLATE CONTENT RAISED BY SULPHUR APPLICATION ON DISEASE CONTROL IN OILSEED RAPE

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## Abstract

Applying sulphur to raise levels of glucosinolates within the leaves and stems of oilseed rape could not fully substitute for fungicide application. However, on sites where sulphur was deficient, yields in some years were not increased by fungicide application or were even lowered by fungicide application, despite a high level of disease. Further work is needed to elucidate the associated changes in metabolism. An implication of the work of relevance to growers is that adequate sulphur nutrition of the oilseed rape plant is essential for efficient utilisation of the fungicide input.

Glucosinolates are sulphur containing secondary metabolites and have been found in every *Brassica* species examined. Anti-fungal effects of breakdown products from glucosinolates have been noted in laboratory studies[1,2] and glucosinolates are thought to be involved with the defence mechanism of the plant against disease[3]. It has been shown that the glucosinolate content within the plant can be raised by increasing sulphur availability[4]. The main objective of the work reported here was to determine whether glucosinolate levels within the plant which had been enhanced by sulphur application, could be utilised to reduce the need for fungicide application.

A series of field trials on oilseed rape was carried out in Scotland, where high yield responses to fungicide application are observed due to routine natural infections by light leaf spot (*Pyrenopeziza brassicae*). Sulphur was applied in the form of potassium sulphate, so that results would not be confounded by the direct fungicidal effect that the alternative foliar application of elemental sulphur may have. Potassium application was balanced for plots receiving low sulphur treatments. Levels of up to 160 kg sulphur $ha^{-1}$ were applied.

At the moderate sulphur site sulphur application had no effect on yield in the three seasons that the trial was carried out, whereas fungicide application was associated with significant increases in yield for two seasons. Data for the 1994/95 season is shown, and significant increases in yield were associated with fungicide application at the 80 and 160 kg S $ha^{-1}$ treatments (Figure 1). It was noted that fungicide had a major effect in reducing disease levels but sulphur had only a minor effect and this was only detected at the early disease assessments. At the low sulphur site, in the absence of applied sulphur, fungicide application had a very small positive effect in two of the four seasons of trials. In the other two seasons, fungicide treatment tended to depress yield when applied to plants which had received no sulphur as shown for the 1993/94 season (Figure 2). Again it was noted that disease levels were lower on fungicide treated plants, but no differences in disease levels were noted according to sulphur application.

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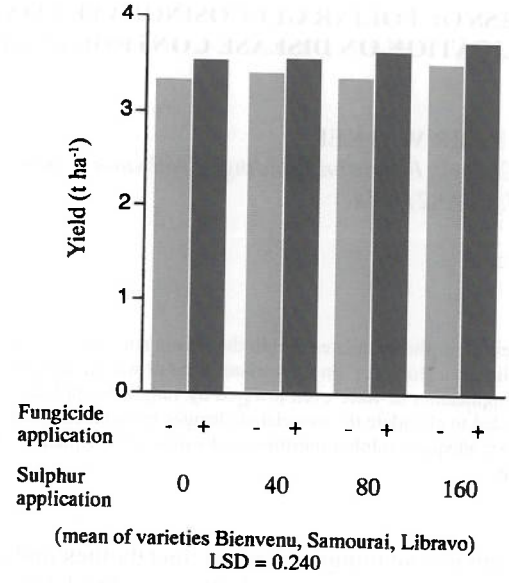


*Fig. 1.* The effect of fungicide application and sulphur application on yield at a moderate sulphur site.

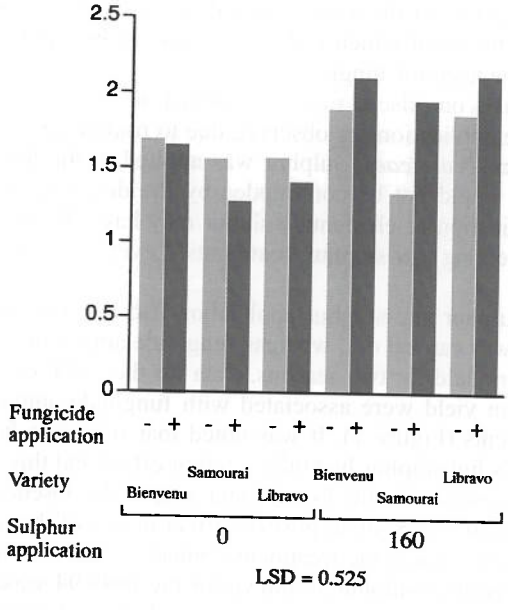


*Fig. 2.* The effect of fungicide application and sulphur application on yield at a low sulphur site.

The trials have demonstrated that raising glucosinolate levels within the plant foliage cannot fully substitute for fungicide application in terms of disease control and subsequent effects on yield. Further work remains to be done to fully elucidate the changes in metabolism associated with the reduction in yield in two of the four seasons when fungicide was applied to oilseed rape on sulphur deficient sites. The studies imply that adequate sulphur nutrition of the oilseed rape plant is essential for efficient utilisation of the fungicide input. Although glucosinolates have been shown to be fungitoxic in laboratory work[1,2] it may be possible that the affect of sulphur application in reducing and delaying disease, which has been seen in both the experiments reported here and previous work[4], may be due to some other additional affect rather than just through foliar glucosinolate enhancement. Sulphur is known to improve fitness of plant and it is not possible at the present time to categorically prove cause and affect.

The present trials and other work in both glasshouse[4] and field[5] experiments, indicates that there does seem to be potential for the use as glucosinolates as biocides. However this is limited according to the glucosinolates presently found within oilseed rape, and it appears that manipulation of the content within the plant can only be partially successful. The development of the biorefinery technique of processing rapeseed will allow the extraction of glucosinolates from Brassica seeds[6] and it is considered that this may offer greater potential for utilising the biocidal potential of glucosinolates. Future work will test this hypothesis.

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# SULPHUR NUTRITION AND ALLIIN CONCENTRATIONS IN *ALLIUM* SPECIES

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## Abstract

Alliin concentrations in foliar tissue of *Allium sativum* and *Allium cepa* are closely related to the S supply of the plants but decrease with aging due to translocation of alliins from leaves to bulbs.

Alliins are characteristic sulphur (S) containing secondary metabolites synthesised by members of the genus *Allium*. The alliin content is an important quality parameter especially with view to organoleptic features and therefore relevant not only in generative but also vegetative plant parts when used in human nutrition (e.g. leek, chives and spring onion). As the S supply has already been shown to have a strong influence on the formation of other S containing secondary metabolites such as glucosinolates[5] the reported research work aimed to quantify this relationship for alliins too. The true relationship between S status and alliin synthesis should be reflected in the alliin concentrations of the photosynthetically active vegetative tissue because the bulbs are storage organs which integrate nutritional effects over time (cf. ref 1). In the present study the influence of the S nutrition on the alliin and isoalliin concentrations in the leaf tissue of garlic and onion are determined at different growth stages.

In a greenhouse experiment 8 onion and 4 garlic plants were grown in 10 kg Kick-Brauckmann pots with sand as substrate. The nitrogen and S treatments were applied in factorial combination in amounts of 0, 50 and 250 mg/pot S and 250, 500 and 1000 mg/pot N. All other nutrients were maintained sufficiently for optimum growth (mg/pot: 103 P, 500 K, 400 Ca, 25 Mg, 10 Fe, 1 Mn, 1 Zn, 0.5 Cu, 0.8 B, 0.05 Mo). The first sampling of leaves was carried out after their full development and the start of bulb sprouting 66 days after sowing and subsequently carried out weekly with 4 harvests in total.

Sample preparation and determination of the alliin (3-(2-propenylsulfinyl)-L-alanine) or isoalliin (3-(1-propenylsulfinyl)-L-alanine) concentration in the leaf tissue of garlic and onion were carried out according to Hoppe et al.[3].

An increasing S supply was related to increasing total S concentrations in garlic and onion leaves (Fig. 1). The distinctively higher S content in garlic leaves also in nil S treatments is due to the fact that the garlic has been planted as bulbs, but the onion as seeds. With raising S concentrations in the leaves the (iso-)alliin content also increased (Figs. 2 & 3). However, the amount of sulphur bound in (iso-)alliins is approximately two times higher in onions than in garlic (Figs. 2 & 3).

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The (iso-)alliin content continuously decreased from the first to the last sampling date in both garlic and onion (Fig. 4). According to Muetsch-Eckner[4] and Cho & Lee[1] this continuous decrease in the alliin content of garlic leaves reflects the translocation of alliins to the bulbs.

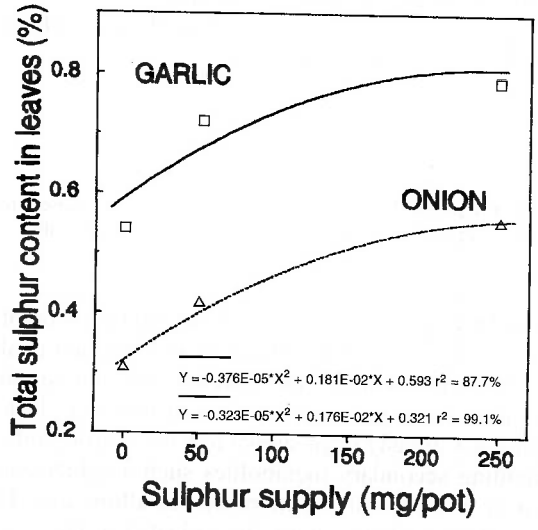


*Fig. 1.* Total S concentrations in garlic and onion leaves as influenced by S supply (mean of all N treatments).

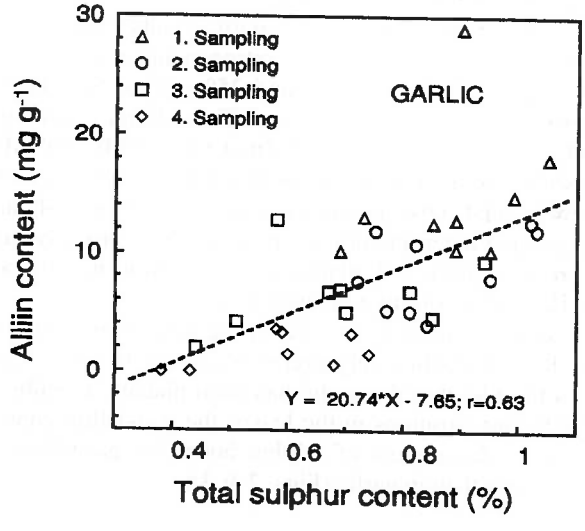


*Fig. 2.* Relationship between total S and alliin content in the leaf tissue of garlic.

The results reveal that the sulphur supply is a major environmental factor maintaining the alliin concentration in *Allium* species. As alliins are responsible for flavour and taste of these plants and the sulphur supply is one of the most variable nutritional growth factors for field grown plants this explains why crops of the same variety but of different origin may differ in taste and flavour.

Moreover sulphur fertilisation of garlic may become an important factor for the maintenance of high alliin contents in the bulbs used for phytomedicinal products.

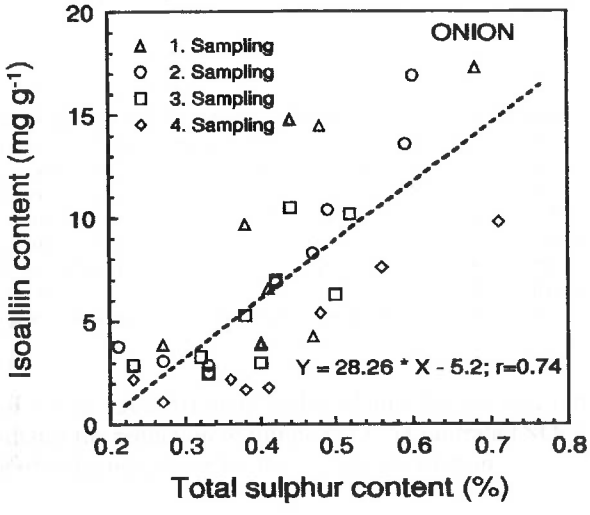


*Fig. 3.* Relationship between total S and isoalliin content in the leaf tissue of onion.

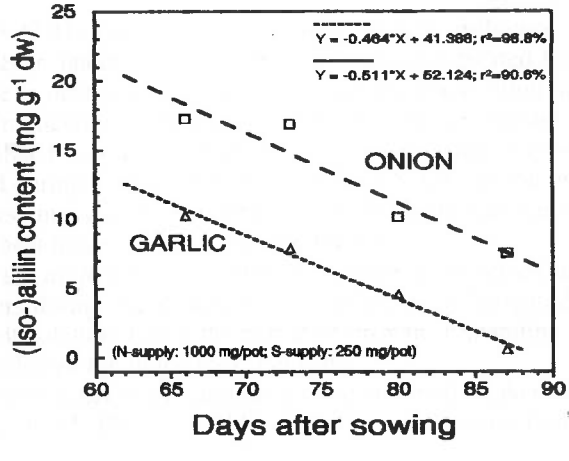


*Fig. 4.* Relationship between growth and (iso-)alliin content in the leaf tissue of garlic and onion.

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# EFFECT OF THIOL COMPOUNDS ON NITRATE REDUCTASE ACTIVITY *IN VIVO* IN WHEAT (*TRITICUM AESTIVUM* L.) LEAVES

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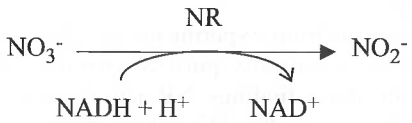
## Abstract

Preincubation of wheat leaves in solutions of thiol compounds viz. cysteine, glutathione, dithiothreitol and ß-mercaptoethanol, enhanced *in vivo* nitrate reductase (NR) activity, considerably. A range of concentrations was used for each of these compounds. NR activity was found highest with glutathione treatment (80% increase over control) followed by cysteine, ß-mercaptoethanol and dithiothreitol. The content of water soluble -SH also increased in the treated leaves. The enzyme activation by the thiol compounds is possibly mediated by NADH production during the thiol reduction of NAD under *in vivo* conditions. The elevated water soluble -SH content possibly increased the NADH generation in the cytosol providing more reducing power to the enzyme. This is supported by the observation that supply of $NAD^+$ together with the above thiols, further enhanced NR activity in the leaf discs. It is suggested that thiol compounds support nitrate reduction *in vivo*, in the leaves of *Triticum aestivum* L.

In the pathway of nitrate assimilation in higher plants, the enzyme nitrate reductase (NR) (EC 1.6.6.1) mediates the reduction of nitrate ($NO_3^-$) to nitrate ($NO_2^-$). NADH is the predominant source of reducing power for this cytosolic reaction[1].




Availability of NADH and the substrate, $NO_3^-$, greatly influence the activity of this enzyme[1,2]. However, under *in vitro* conditions NR is inactivated by NADH, especially when the enzyme is deprived of nitrate[3]. The enzyme inactivation under *in vitro* conditions by thiol compounds viz. dithiothreitol (DTT) dithioerythreitol (DTE), and β-mercaptoethanol (BME) was also reported[4]. This inactivation was shown to be due to NADH produced during thiol-mediated reduction of $NAD^+$. In the present investigation, we report that cysteine, glutathione (GSH), DTT and BME have an enhancing effect on NR activity in wheat leaves under *in vivo* conditions.

Wheat (*Triticum aestivum* L.; cv. HD 204) plants were raised in plastic pots under natural conditions during the growth season. Plants were irrigated with half strength Hoagland nutrient solution during the period of growth. Expanding leaves from 30-day old plants were selected for all the measurements.

Leaves cut under water were carefully placed in small beakers containing the solutions of cysteine, GSH, DTT and BME over a range of concentrations. The beakers,

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wrapped with black paper, were placed under light (500 μE $m^{-2}$ $s^{-1}$) at 25°C. $NO_3^-$ (5 mM) was added in all the treatment solutions including that of the control. Leaf samples collected at 60 and 180 min. after incubation were assayed for NR activity, *in vivo* and *in vitro*, and for water soluble -SH content.

Nitrate reductase activity *in vivo* was determined according to Klepper *et al*[5]. $NAD^+$ (500 μM wherever indicated) was included in the assay medium prior to vacuum infiltration. *In vitro* NR activity was determined according to the method of Hageman and Hucklesby[6]. Three replications from each treatment were taken for the above assays.

Water soluble -SH content was determined in leaves homogenized in 0.15% sodium ascorbate solution (w/v; 5 ml/g fw) at 0°C. The homogenate was filtered through one layer of Mira cloth and the filtrate centrifuged at 30,000 g for 15 min. at 4°C. To 1 ml of the clear supernatant, 1.2 ml of 0.2 M potassium phosphate buffer (pH 7.0) was added according to the method described by De Kok *et al*[7]. The yellow coloured product was measured at 412 nm spectrophotometrically and the absorbance corrected for the colours of the supernatant and DTNB. Three measurements were made from each treatment.

A range of concentrations of thiol compounds were used to select an appropriate concentration for further use. Highest NR activity *in vivo* was obtained with 5 mM cysteine, 20 mM GSH, 1mM DTT and 0.5 mM BME. Glutathione, amongst the various thiol compounds, exerted maximum influence in enhancing *in vivo* NR activity (80% over control) in wheat leaves (Table 1). Inclusion of $NAD^+$ in the assay medium further enhanced the activity in the leaves preincubated with the thiol compounds (Table 2). NR activity *in vitro*, however, declined as a result of thiol treatment in all cases (Table 1). NR can be reversibly inactivated by NADH in the absence of nitrate[8]. A similar type of NR inactivation by thiol compounds has been reported in spinach[9]. Inclusion of $NAD^+$ along with the thiol compounds was further shown to enhance the inactivation of the enzyme[4]. Such an activation was shown to be mediated through NADH produced by the enzymatic reduction of $NAD^+$ by thiol compounds.

The above reports pertained to the results obtained from experiments in which NR activity *in vitro* was assayed using crude extracts of partially purified enzyme. Our observations on *in vitro* NR activity agree with the above findings. NR activity assayed under *in vivo* conditions, however, gave contradicting results. The enhancement of *in vivo* NR activity of wheat leaves saturated with nitrate and thiol compounds was further increased with the addition of $NAD^+$. It is possibly mediated by NADH production during thiol reduction of $NAD^+$ under *in vivo* situation. Elevated levels of -SH content in the leaves treated with the thiol compounds possibly increased the NADH concentration in the cytosol, thus providing more reducing power to the enzyme. This is supported by the observation that provision of $NAD^+$, along with the thiol compounds, enhanced the enzyme activity when compared to that discernible with the thiol compounds alone. Similar results were obtained in experiments using the leaves of *Brassica campestris*, in our laboratory. Thiol compounds were shown to be involved in the reduction of $NAD^+$ to NADH resulting in the inactivation of nitrate reductase *in vitro*[4]. NR inactivation by elevated NADH concentration under *in situ* conditions, however, is less likely as excess NADH could be dissipated via the mitochondrial electron transport chain or by different metabolic reactions utilizing NADH.

*Table 1.* Effect of thiol compounds on nitrate reductase (NR) activity and water soluble -SH content in wheat (*Triticum aestivum* L.) leaves. The data represent the mean (±SE) of three replicates

| Treatment | NR activity | | | | Water soluble -SH | |
|---|---|---|---|---|---|---|
| | *in vivo* (μmol $NO_2^-$ $g^{-1}$ fw $h^{-1}$) | (%) | *in vitro* (μmol $NO_2^-$ $g^{-1}$ fw $h^{-1}$) | (%) | (μmol $g^{-1}$ fw) | (%) |
| Control ($H_2O$) | 4.13 ± 0.09 | 100 | 9.06 ± 0.12 | 100 | 0.68 ± 0.003 | 100 |
| Cysteine (5 mM) | 5.82 ± 0.03 | 141 | 8.81 ± 0.10 | 97 | 1.05 ± 0.007 | 154 |
| Glutathione (20 mM) | 7.43 ± 0.14 | 180 | 7.07 ± 0.06 | 78 | 1.20 ± 0.009 | 176 |
| Dithiothreitol (1 mM) | 5.02 ± 0.07 | 122 | 7.41 ± 0.10 | 82 | 1.01 ± 0.005 | 148 |
| β-mercaptoethanol (0.5 mM) | 5.17 ± 0.04 | 138 | 7.25 ± 0.09 | 80 | 0.87 ± 0.005 | 128 |

*Table 2.* Influence of thiol compounds in combination with $NAD^+$ on *in vivo* nitrate reductase (NR) activity in wheat (*Triticum aestivum*) leaves. The data represent the mean (+ SE) of three replications

| Treatment | *In vivo* NR activity (μmol $NO_2^-$ $g^{-1}$ fw $h^{-1}$) | (%) |
|---|---|---|
| Control ($H_2O$) + $NAD^+$ | 4.75 ± 0.05 | 100 |
| Cysteine + $NAD^+$ | 7.09 ± 0.18 | 149 |
| Glutathione + $NAD^+$ | 10.24 ± 0.21 | 215 |
| Dithiothreitol + $NAD^+$ | 6.73 ± 0.15 | 141 |
| β-mercaptoethanol + $NAD^+$ | 6.68 ± 0.11 | 140 |

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# CHANGES IN NUTRIENT COMPOSITION, METABOLITE CONCENTRATIONS AND ENZYME ACTIVITIES IN SPINACH IN THE EARLY STAGES OF S-DEPRIVATION

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## Abstract

As the $SO_4^{2-}$ content of expanding leaves declined, during $SO_4^{2-}$-deprivation, the concentrations of arginine, glutamine and $NO_3^-$ increased sharply, indicative of disturbed $NO_3^-$ reduction and protein synthesis. Despite this, significant de-repression of S-assimilatory enzyme activities was seen only in roots and not in young leaves.

There are interactions between sulphur and nitrogen metabolism[1,2], which have led to suggestions that the transport[3] and metabolic pathways[4] may be co-regulated. If this is so, it is clear that this co-regulation is imperfect since it is widely recognised that, particularly during sulphur-deficiency, there may be large accumulations of unprocessed nitrate or amino compounds in leaves[5,6]. Although nitrate uptake and assimilation are lowered by S-deficiency, the data outlined in this paper show that inefficient utilisation of N occurs as an early consequence of sulphate-deprivation (-S). This raises questions about the putative role of metabolite repressors in regulating the two pathways of N and S assimilation.

Spinach plants were grown hydroponically in bright light (500 μmol $m^{-2}$ $s^{-1}$ PAR). In the pre-experimental period the plants were supplied adequately with all nutrients, including $SO_4^{2-}$ at 1.5 mol $m^{-3}$. When approximately 5 weeks old, half of the plants were transferred to a solution lacking $SO_4^{2-}$. Young (expanding) and mature (mostly completed expansion) leaves and roots were sampled at regular intervals over 6-8 days in 3 similar experiments. Effects on plant growth were small during this period but young leaves became somewhat yellow-green in colour.

The sulphate concentration declined at different rates in tissues during -S; the effects were in the order root > young leaf > mature leaf. In young leaves the earliest effects of -S were the marked accumulation of arginine (Arg), glutamine (Gln) and nitrate (Fig. 1). The effect on Arg was less marked in mature leaves and occurred somewhat later; the effect on Gln was not found in mature leaves. The $NO_3^-$ accumulation occurred despite lowered $NO_3^-$ influx into, and transport from the roots (data not shown). Nitrate reductase activity (NRA) *in vitro* declined in young leaves but in mature ones changes in NRA could not be separated from a decline accompanying leaf ageing. The decreased NRA in young leaves was accompanied by a reduced number of NR and nitrite reductase (NiR) gene transcripts if the results were assessed on the basis of leaf weight or area (data not shown). However, the total RNA abundance in -S leaves declined at about the same rate as the NR and NiR mRNAs so their specific abundance was unchanged. Quantitatively, the effects on the accumulation of $NO_3^-$ and amino compounds (Fig. 1) were much more sensitive indicators of disturbed N utilisation than NRA or mRNA.

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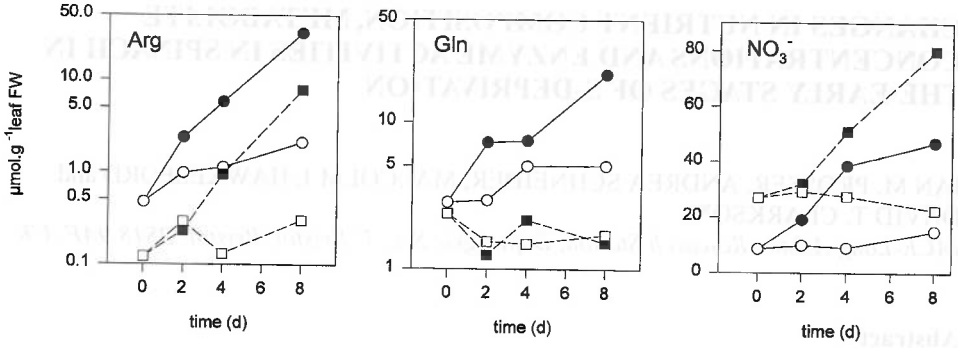


*Fig. 1.* Metabolite concentrations in young (circles) and mature (squares) leaves of Spinach grown with (open symbols) or without (solid symbols) sulphate in the external medium. Log scales are used in plots of the arginine (Arg) and glutamine (Gln) data.

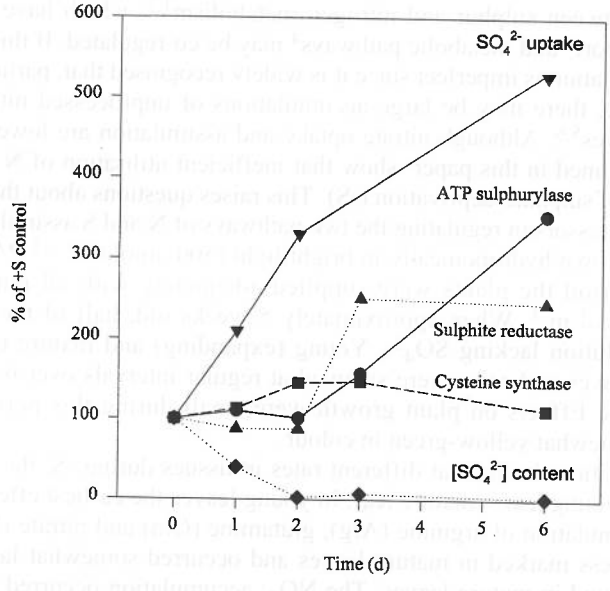


*Fig. 2.* Enzyme activities, sulphate influx and concentration in roots of spinach deprived of S for various periods. Values are % of +S control.

Sulphate influx into roots responded more quickly to -S than any other parameter measured, and occurred several days in advance of changes in the *in vitro* activities of enzymes of S-assimilation. In roots, it was only when the tissue [$SO_4^{2-}$] had fallen to below 10% of its initial value that positive effects became apparent on the activities of sulphite reductase, ATP sulphurylase and cysteine synthase (Fig. 2). In leaves, no effects on these enzyme activities were seen, even though there was a substantial fall in [$SO_4^{2-}$], especially in young leaves.

It is a paradox that S-deprivation seemed to have a more immediate effect on the assimilation and utilisation of N than of S in expanding leaf tissue. The relatively trivial

effect, on the transcription of the NR gene, of a more than ten-fold increase in the Gln concentration of young leaves, either casts some doubt on the role of Gln as the cardinal metabolite repressor in the N-regulatory circuit, or implies very effective sequestering of surplus Gln in the leaf vacuoles.

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# A NEW X-RAY BASED TECHNIQUE TO TRACK UPTAKE AND METABOLISATION OF FOLIAR APPLIED SULPHUR SOURCES

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## Abstract

A new technique based on X-ray fluorescence spectroscopy (X-RF) which enables the non-destructive tracking of uptake and metabolism of foliar applied S compounds is introduced. Application of the method to *Brassica napus* leaves showed that (a) foliar applied elemental S needs oxidation prior to uptake and (b) that lower rates of transport from foliar applied elemental S into the cytosol results in immediate metabolism.

The efficiency of foliar applied sources of plant nutrients depends on conversion processes on the leaf surface, the transport through the cuticule into the cytosol and the metabolism of the nutrient in the cytosol. Following this complex processes by common methods of chemical analysis and tracer techniques is very difficult, because they need to employ mechanical fractionation to map spatial separation. This paper reports details of a non-destructive method for S sources employing two physico-chemical characteristics of X-ray fluorescence radiation derived from S atoms which have a penetration depth less than the average thickness of leaves but greater than the thicknes of the cuticule and shifts of the S-Kα peak depending on the oxidation number.

*Foliar applications*: 4 µl $cm^{-2}$ of solutions containing 2.5% S as $MgSO_4$ $7H_2O$ (Epsom salt) and elemental S (THIOVIT) were applied to the upper surface of a *Brassica napus* leaf by means of a very soft small brush, taking care to avoid injuries to the leaf and contamination of the lower leaf surface. The amount of S applied in this way corresponds to a field treatment of 10 kg $ha^{-1}$ S. The fertiliser solutions contained 0.01% of a common detergent ("PRIL"). The time to conduct each treatment did not exceed 2 minutes. Time "0" in figure 1 corresponds to the end of the particular treatment procedure. Each marker in figure 1 represents the mean of 4 replicates.

*Sampling and sample preparation:* 15, 30, 60, 120 and 180 minutes after finishing the foliar application, the upper leaf surface was first wiped dry by means of a soft paper tissue. Then a disk of 4 cm diameter was punched out of the leaf and transferred to a sample mount exposing the lower, untreated side to the X-ray beam of an X-ray fluorescence spectrometer (see below). To keep the leaf disc in position during measurement, it was backed by a 5 mm thick wax pellet followed by a 50g brass weight.

*Detection of foliar uptake and identification of reduced and oxidised S in leaves* was achieved by X-ray fluorescence spectroscopy using the phenomenon of wavelength shifts in the S-Kα radiation caused by different oxidation numbers of $S^3$. Due to mass absorption S-Kα radiation in dry plant material it is only collected from a sample depth not more than 0.1-0.15 mm[6]. Fresh leaves have higher oxygen concentrations which again increase the leaves mass absorption coefficient and reduce the depth from which

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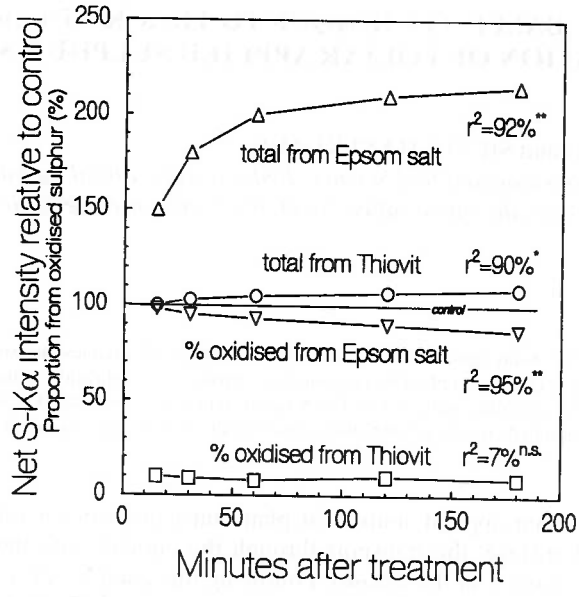


*Fig. 1.* Relative intensities of S-Kα intensities measured from the lower surface of *Brassica napus* leaves after application of sulphur with $MgSO_4$ $7H_2O$ (Epsom salts) and elemental sulphur (Thiovit).

S-Kα radiation can be expected to not more than 0.08-0.1 mm. Thus, if S is only applied to one leaf surface, it should not be detectable by X-ray fluorescence spectroscopy from the opposite side, unless it is taken up via the epidermis and distributed within at least 50% of the upper portion of the leaf volume. This hypothesis has been checked by transferring untreated leaf disks to the sample mount and backing them with either a 5 mm thick S free wax disk or a 5 mm thick disk prepared from solid molten elemental S. No difference in S-Kα radiation between the two backing disks was found, which proves that as long as S is not taken up by the leaf and is just sticking to the leaf surface it can not be detected by this method. On the other hand fresh leaves collected from plants with different S supplies showed different S-Kα intensities when analysed in the way described above, which proves that S allocated within the leaf matrix delivers S-Kα radiation depending on concentration. At present there is no way to calibrate these measurements and therefore results have to be presented as relative changes in the S-Kα intensities collected from the different treatments. Although quantitative assessments can only be made relatively, the great advantage of the proposed method is that it works on undamaged leaf structures and thus avoids artefacts arising from chemical extraction and fractionation or mechanical cutting.

*Instrument and settings*: PHILIPS PW 1400 X-ray fluorescence spectrometer, Cr-tube maintained at 50 kV and 40 mA, wavelength dispersion on PE crystal, flow counter maintained with $Ar/CH_4$, identification of S-species specific peak shifts for the S-Kα followed the procedures described by Pinkerton et al.[5]. The instrument needed to be operated at normal atmospheric pressure and purged with helium in order to avoid cell breakage caused by the vacuum.

After foliar application, intensities derived from total S increased much faster with sulphate applied as Epsom salts, than with elemental S applied as Thiovit (Fig. 1). This indicates clearly that the latter was moving at lower rates through the cuticule. There are

two major explanations for this observation: either the microbiotic conversion to the obviously much faster moving sulphate is limited[2], or the diffusion of unchanged elemental S through the cuticule is hampered because it is in fact a non water-soluble compound occurring in larger, mostly cyclic structures[8]. Even if elemental S moved inside the cell, it needs oxidation to sulphate[4] because a direct entrance of cyclic $S_8$ structures or other condensation products into S metabolism are not likely to occur. Also as molecular aggregations of S atoms like cyclic $S_8$ structures are xenobiotics for higher plants, a "chloroplast specific enzymatic oxidation process" as proposed by Jolivet[4] would run only slowly. Compared to the control approximately 10% higher levels from oxidised S were found in Thiovit treated leaves (Fig. 1). The increased amounts of oxidised S detected in leaves immediately after treatment with Thiovit remained at a nearly constant level over the whole observation time without any significant trend (Fig. 1). All these facts support the hypothesis that any significant contribution of foliar applied elemental S to the S nutrition of the plant requires prior oxidation on the leaf surface and that this process is the main factor limiting its uptake. The initial relation of sulphate concentrations in the cytosol and in the applied solution was approximately 1:500. The intensity of total S-Kα, representing the transport rate of sulphate from Epsom salts declined with time which indicates a saturation of the cytosol in the sulphate treated leaves (Fig. 1). This could either indicate a saturation effect or the beginning of an "overflow" process by which a particular leaf starts to export sulphate when it exceeds a certain level[1,7].

As a matter of metabolic reduction, the intensities of oxidised S collected from Epsom salts treated leaves decreased within the same time. The relative decrease of oxidised S intensities collected from Epsom salts treated plants is in the same range as the relative increase of intensities collected from total S in Thiovit treated leaves (0.05-0.06% $min^{-1}$, Fig. 1). Both obviously reflect the rate at which sulphate is metabolised in the cell. Together with the fact that no increase in oxidised S intensities from Thiovit treated leaves were detected, this finding would imply that all S derived from foliar applied Thiovit is immediately metabolised.

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# IMPORTANCE OF SULPHUR IN UK AGRICULTURE

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## Abstract

Incidence of sulphur deficiency has increased considerably in UK crops in the last decade. A risk assessment model predicts that S deficiency areas will increase much further as inputs from atmospheric deposition continue to decline. Yield responses to fertiliser S have been demonstrated in different crops, whereas the effects on quality can be either positive or negative.

Sulphur is an essential element for plant growth and development. Shortage of S results in yield losses and possibly poor quality. The optimum amount of S required by crops is determined mainly by crop species and yield potential. Recent studies suggest that oilseed rape requires about 16 kg S $ha^{-1}$ to produce 1 tonne $ha^{-1}$ seed yield, whereas for cereals about 2-3 kg S $ha^{-1}$ is taken up per tonne of grain produced[1]. Therefore, average oilseed rape and cereal crops require approximately 50 and 20 kg S $ha^{-1}$, respectively.

In the past, industrial pollution ensured an adequate S supply for crops in most of Britain. However, due to the concerns about the effects of acid rain on natural ecosystems which exist on poorly buffered soils and catchments, steps have been taken to reduce the emissions of $SO_2$, both in the UK and other European countries. Total emissions of $SO_2$ in the UK decreased from 6.4 million tonnes in 1970 to 2.8 million tonnes in 1994, and are set, according to an European agreement, to decrease much further to less than 1.5 million tonnes by 2005 and 1 million tonnes by 2010[2]. At present atmospheric S deposition over three quarters of Britain is less than 20 kg $ha^{-1}$ $year^{-1}$, which is below the minimum that crops need.

Decreased S inputs are reflected in changes in crop S status. The mean concentration of S in the total of 793 wheat grain samples collected representatively from Britain in 1992/93 was 1.35 mg $g^{-1}$, and this was much smaller than the mean of 1.72 mg $g^{-1}$ for the samples of 1981/82[3]. Sulphur deficiency has become widespread in UK arable crops. The increased incidence of S deficiency in recent years has been more pronounced in England and Wales than in Scotland. The latter has had cleaner air and associated reports of S deficiency for a longer period. Between a quarter and a third of field experiments conducted during 1986-1995 showed significant yield responses to the addition of S[1]. Because of their high S requirement, oilseed rape and multi-cut grass were the most responsive crops, with yield increases in the range of 10-327% and 5-134%, respectively, whereas cereals produced yield increases of 4-40%. Severe S deficiency also resulted in lower oil contents in rapeseed[4]. Important interactions between N and S supply on seed yields were evident in field experiments[1,5].

The S nutrition of a crop often has a strong influence, not always positive, on the quality of the produce, because of its essential role in the synthesis of amino acids, proteins and some secondary metabolites. It has been shown that S nutrition plays an impor-

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tant role in breadmaking quality of wheat, by affecting the spectrum of storage proteins, and the strength and extensibility of dough[6]. The effects of S on breadmaking quality have recently been investigated under field conditions in the UK. Preliminary results showed that addition of S fertiliser increased loaf volume significantly[1].

In contrast, excessive S supply can result in a high concentration of glucosinolates in rapeseed. Glucosinolate concentration is usually higher at high S than at low S sites. However, addition of S fertiliser tends to increase glucosinolate content much more under S-deficient than under S-sufficient conditions[5,7]. In some cases, the increases were 2-3 fold, resulting in a glucosinolate level exceeding the current threshold value of 18 μmol $g^{-1}$ for seeds to be used for sowing in the following year. Nitrogen and S nutrition was found to interact strongly on both the total concentration and the profile of individual glucosinolates[8]. The benefits of S on seed yield and the potential adverse effect on quality of rapeseed meal are difficult to reconcile, but applying more S than required by oilseed rape crop should be avoided.

A number of factors interact to determine whether S deficiency is likely to occur in any crop. These include the inputs of S from the atmosphere, soil S supply, likelihood of leaching, and crop requirement and growth pattern. Because detailed site specific information about S mineralisation and leaching is not available, we have used a qualitative computer model to predict the extent of present and future S deficiency in Britain, using data of atmospheric S deposition, various soil characteristics, rainfall and crop requirement as inputs[9]. The model predicts that currently 11% of the total land area in Britain is at high risk of S deficiency for cereal crops, and a further 22% at medium risk. For oilseed rape, 33 and 22% are at high and medium risk, respectively. Meeting the $SO_2$ emission target of the year 2003 would increase the high and medium risk areas for cereals to 23 and 27%, and for oilseed rape to 50 and 20%, respectively. The prediction highlights the importance of S as a plant nutrient in UK agriculture.

The main forms of S fertilisers used are sulphates and elemental S. Limited comparative field studies in the UK indicate that in high rainfall conditions elemental S may be more effective than sulphate, because of higher leaching losses of the latter[1]. Under drier conditions, sulphate is prefered because it is more readily available to crops.

Great effort has gone into the study of the negative effects anthropogenic S deposition on natural ecosystems. However, now more studies are required to look at the S cycling and the availability of soil S to crops under deficient conditions. More reliable diagnostic methods are needed to identify S deficiency at the earliest possible stage of crop growth. The effects of S on crop quality need to be investigated further under realistic field conditions.

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# DIAGNOSIS OF SULPHUR DEFICIENCY IN WHEAT

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## Abstract

Glutathione (GSH) in wheat leaves showed the most sensitive response to S supply in a pot experiment, whereas in a field experiment the concentration of sulphate was more sensitive than GSH. Both sulphate and GSH concentrations correlated well with dry mater yields of two varieties of wheat in the pot experiment, and could be used as a diagnostic index for S deficiency. The results are being tested in field experiments.

Sulphur (S) deficiency has become increasingly widespread in many crops in western Europe[1,2]. Reliable diagnosis of S deficiency is needed to avoid yield losses and to ensure efficient use of S fertilisers. Diagnostic indices such as total S, sulphate-S and N:S ratio have been proposed, but there is no agreement as to which index gives the best result. Although glutathione (GSH) accounts for a small portion of the total S in plants, it represents a major pool of metabolically available S and may also act as a signal to regulate sulphate uptake and transport[3]. The objective of this study was to investigate responses of various chemical and biochemical parameters of wheat to the addition of S under controlled or field conditions.

In a pot experiment using a S-deficient soil, growth of wheat was greatly improved by S. Fig. 1 shows that the relative response to S (taking the $S_0$ treatment as 1) was greatest with GSH by a factor of 14 in the 50 mg S treatment. Sulphate and total S concentrations responded by a factor of 8 and 4, respectively. In contrast, cysteine (CYS) level was little changed by the S treatments. Similar patterns were observed at two N levels and with two varieties.

Relative dry matter (DM) yields of wheat shoots at the Zadok's GS37 growth stage were plotted against the concentrations of sulphate and GSH (Fig. 2). The breadmaking variety Hereward and non-breadmaking variety Riband showed similar relationships. Critical values for sulphate in shoots and GSH in the uppermost developed leaves were about 200 mg $kg^{-1}$ DM and 250 nmol $g^{-1}$ fresh weight (FW), respectively.

Some preliminary results from a field experiment are shown in Table 1. Application of 40 kg S $ha^{-1}$ increased chlorophyll content significantly ($p<0.01$) in both varieties. There were also significant effects on the concentrations of total S, sulphate-S and GSH. Sulphate-S showed the greatest response, followed by GSH and total S. Sulphate-S concentrations in the $S_0$ treatment were below the critical value obtained in the pot experiment. For GSH, Riband had a concentration lower than the critical value when no S was applied, whereas Hereward contained higher than the critical concentration. It must be pointed out, however, that the samples were taken at an earlier growth stage than those for the pot experiment.

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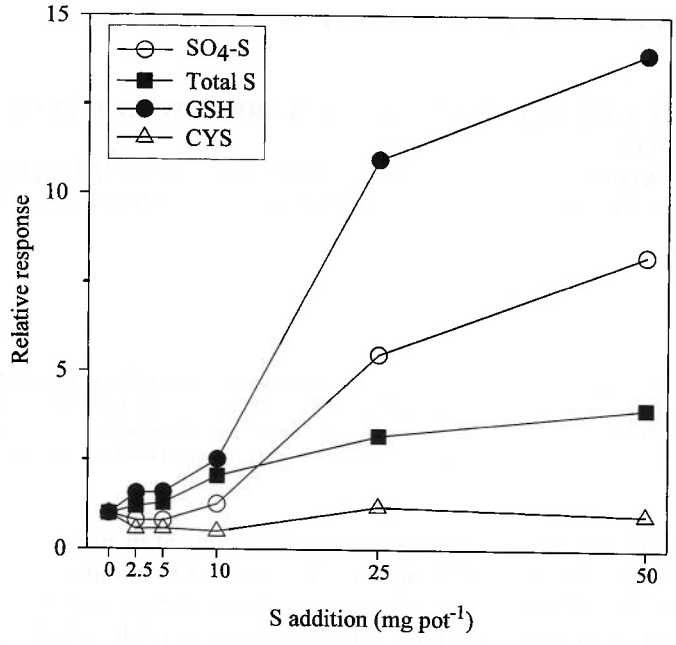


*Fig. 1.* Relative responses of total S, sulphate, glutathione and cysteine to S addition. The results were for the variety Riband grown under controlled conditions. Plants were sampled at flag leaf stage (Zadok's GS37). Uppermost fully developed leaves were analysed for GSH and CYS concentrations using HPLC, and whole shoots were determined for total S and sulphate using ICP and ion chromatograph, respectively. For details see Zhao *et al.*[4].

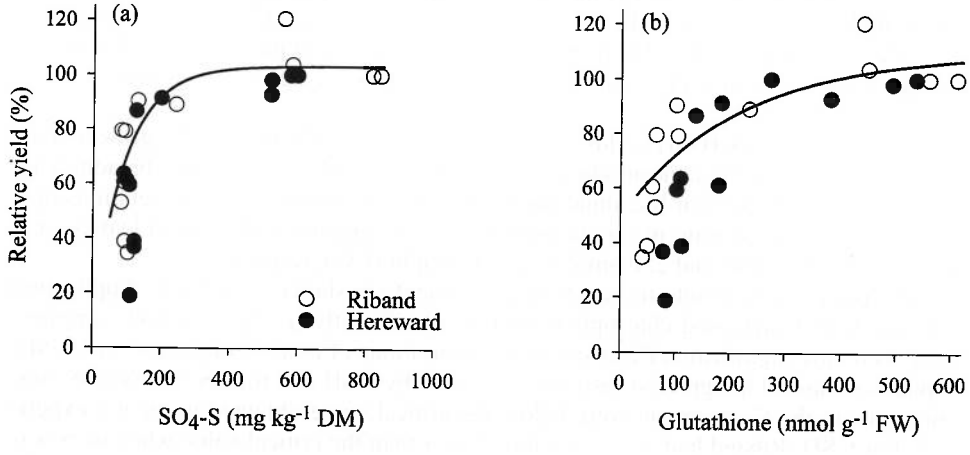


*Fig. 2.* Relationships between relative DM yield and sulphate-S and glutathione concentrations at GS37 under controlled conditions.

*Table 1.* Effects of S addition on the concentrations of chlorophyll, total and sulphate-S, and GSH of two wheat varieties grown in a field (Woburn 1995, early stem extension, Zadok's GS 31)

| Variety and S treatment | | Chlorophyll meter reading | Shoot sulphate-S (mg $kg^{-1}$ DM) | Shoot total S (mg $kg^{-1}$ DM) | Leaf GSH (nmol $g^{-1}$ FW) |
|---|---|---|---|---|---|
| Riband | $S_0$ | 45.0 | 155 | 2300 | 215 |
| | $S_{40}$ | 55.4 | 1346 | 4317 | 687 |
| Hereward | $S_0$ | 43.4 | 169 | 1976 | 353 |
| | $S_{40}$ | 53.2 | 1639 | 4363 | 962 |
| SED (11 DF) | | 1.07 | 69.8 | 164.8 | 59.2 |

In conclusion, GSH was the most sensitive parameter to S status in wheat under controlled conditions, whereas in the field experiment shoot sulphate showed the greatest response to S addition. Both may be used to diagnose S deficiency in wheat. More research is needed to investigate the variations of GSH as influenced by S nutrition and other environmental factors.

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# APPENDIX

## APPENDIX 1. *"SULPUR"*

A long continuing problem for those in the field is the alternate spellings of the element S. We were pleased that the Vice Chancellor of the University of Newcastle upon Tyne – Mr James Wright – agreed to come to welcome and meet the participants in the Workshop. We invited him, as a classical scholar, to comment on the etymology of the name represented chemically as "S", hoping to obtain a rational basis for a universally accepted spelling. He told us that while "phosphorous" has an etymology that points us to the usual spelling, the original Latin word for the yellow substance found in fumaroles and volcanoes is "*sulpur*". This is no help! The current spelling problem has therefore no neat etymological solution, but rather arises from two traditions developing side by side over the centuries.

(See also Cram & Cram, 1997, Nature 385, 196)

## APPENDIX 2. TYNESIDE SULPHUR SONG

(After "Die Forelle")

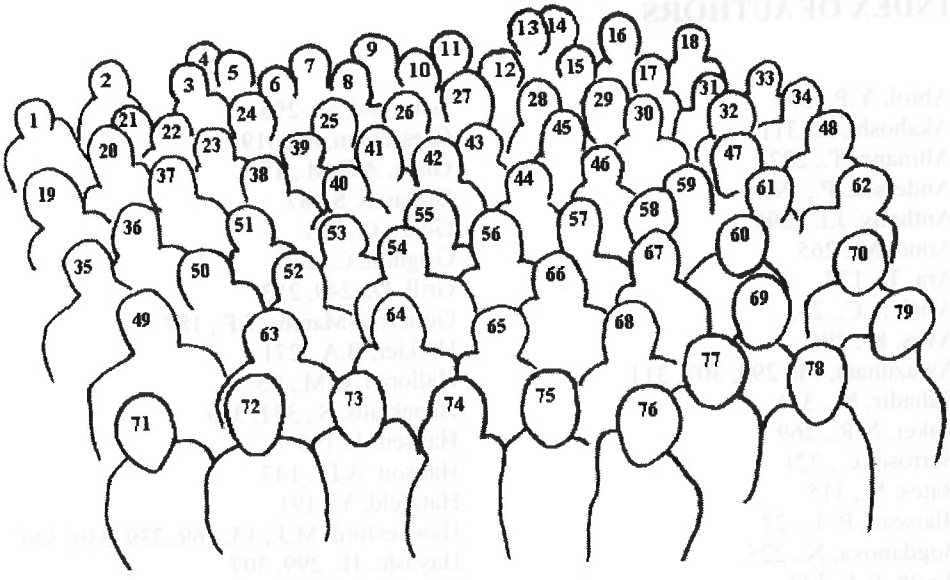
I stand beside a river
that sparkles on its way,
The Tyne flows to the city
where Geordies love to play.
In former days they built a bridge
of steel to cross the Tyne,
Along the banks, great ships were built
for every shipping line.

Alas, this sparkling river
with currents strong and fast
Got cluttered up with rubbish
the odd curd sliding past.
But lo, the city's council
took action, wise and strong
To purify the river,
and so prolong this song.

Now, John Cram and his cohorts
thought this an excellent place
For those who work with sulphur
to gather, face to face,
To hear how plants make cys-te-ine
and construct gluco-sin-o-lates
And redistribute sulphur
at various sorts of rates.

We must tell our politicians
that sulphur holds the key of life
Without research on sulphur
the world will be in strife.
To those who organised this workshop
our thanks for asking us along.
Our thanks to Schubert for the music
for this Tyneside sulphur song.

John Anderson
Newcastle, April, 1966



1. A.D. Hanson; 2. Ch. Brunold; 3. R. Höfgen; 4. T.J. Higgins; 5. J.L. Wray; 6. B. Touraine; 7. D. Zhang; 8. B.J. Kreuzwieser; 9. M. Bucher; 10. J.R. Howarth; 11. A. Rose; 12. R. Hell; 13. G. P. Telkamp; 14. A. M. Poortinga; 15. 16. J.-C. Davidian; 17. T. Fujiwara; 18. K. Müntz; 19. P.M. Mullineaux; 20. L.J. De Kok; 21. P.L. Graziano; 22. M. Burgener; 23. S. Youssefian; 24. D. Klonus; 25. S. Haneklaus; 26. P.F. Johannesen; 27. W.H.O. Ernst; 28. T. Leustek; 29. M. Otte; 30. H. Rennenberg; 31. T.J. Bach. 32. J.D. Schwenn; 33. K. Saito; 34. W.J. Cram; 35. N. Bates; 36. A. Schneider; 37. R. Walsgrove; 38. B. Will; 39. Y. Surdin-Kerjan; 40. D. Grill; 41. H. Hesse; 42. T. Wüstenhagen; 43. J.J. Vidmar; 44. A. Giesemann; 45. M.J. Hawkesford; 46. M.A. Fitzgerald; 47. M. Droux; 48. E. Schnug; 49. S. Ceccotti; 50. L. Du; 51. H. Flückiger; 52. G. Criessen; 53. A.A.G. Lappartient; 54. J.M. Vega; 55. M. Schulte; 56. Y. Hatzfeld; 57. U. Pultke; 58. T. Ara; 59. C.E.E. Stuiver; 60. E.J. Evans; 61. D.T. Clarkson; 62. J. Nieder; 63. C. Herschbach; 64. I. Kranner; 65. M. Yamazaki; 66. K.R. Gayler; 67. K.C. Lakkineni; 68. F. Zhao; 69. J. Sekiya; 70. E. Martinoia; 71. M. Awazuhara; 72. A. Arisi; 73. C. Gotor; 74. M.Y. Hirai; 75. H. Kim; 76. M. Aono; 77. I. Stulen; 78. N.I. Shevyakova; 79. B.A. Halkier.

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